IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

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Applicant	:	Jan Bergstrom	
Filed	:	April 13, 2006	
TC/A.U.	:	1797	
Examiner	:	Katherine M. Zalasky	
Docket No.	:	PU0378	
Customer No.	:	22840	

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Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

August 5, 2009

APPEAL BRIEF

Sir:

Appellants submit this Appeal Brief, appealing from the February 9, 2009 final rejection and the June 15, 2009 Advisory Action of the Examiner, finally rejecting claims 1, 3-9 and 11-15 in the captioned application. The Notice of Appeal was filed on June 9, 2009, which contained authorization to charge the "Appeal Fee" to Appellants' Deposit Account.

Real Party in Interest

GE Healthcare Bio-Sciences AB, the assignee and owner of the captioned application, is the real party in interest to this appeal.

Related Appeals and Interferences

There are no other appeals or interferences related to the instant appeal.

Status of Claims

Claims 2, 10, 19 and 53 have been canceled. Claims 16-18, 20-52 and 54-55 are withdrawn from consideration as belong to non-elected inventions. Claims 1, 3-9 and 11-15 are the only pending claims subject to examination before the U.S. Patent and Trademark Office. Claims 1, 3-9 and 11-15 are finally rejected and constitute the claims under appeal. A copy of these claims is appended hereto.

Status of Amendments

Appellants submit that there are no other outstanding amendments with regard to the captioned application.

Summary of Claimed Subject Matter

The instant invention relates to a novel separation matrix. The separation matrix comprises a porous support that includes porous particles and ligands coupled to the surfaces of the porous support. The ligands provide at least one chemical gradient in the support. The at least one chemical gradient is a continuous and smooth gradient, and the chemical gradient(s) extend between the center and the exterior surface of each porous particle. Claim 1 is the only independent claim. The separation matrix of claim 1 is comprised of a porous support and ligands coupled to the surfaces of the porous support (page 8, lines 27-28). The ligands provide at least one chemical gradient in the support (page 8, lines 28-29), which chemical gradient is a continuous and smooth gradient (page 13, lines 4-5). The claimed porous support includes porous particles (page 19, lines 11-12), and the chemical gradient(s) extend between the center and the exterior surface of each porous particle (page 19, lines 12-13).

Ground of Rejection to be Reviewed on Appeal

Whether claims 1, 3-9 and 11-15 are properly rejected under 35 U.S.C. §103(a) as being unpatentable over Bergstrom et al. (US 6,426,315), as evidenced by Carlsson et al. (US 6,528,322), Gleason et al. (US 5,561,097) and Velander et al. (US 5,977,345).

Argument

Claims 1, 3-9 and 11-15 are not properly rejected under 35 U.S.C. §103(a) as being unpatentable over Bergstrom et al.

Claims 1, 3-9 and 11-15 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Bergstrom et al., as evidenced by Carlsson et al. In the advisory action, the Examiner also cited Gleason et al. and Velander et al. as further evidence supporting the rejection.

Appellants first submit that the claimed invention relates to a separation matrix comprising a porous support and ligands coupled to the surfaces of porous support, characterized by the presence of at least one chemical gradient in the support provided by the ligands. The at least one chemical gradient is defined as a continuous and smooth gradient. The porous support includes porous particles, and the chemical gradient(s) extend between the center and the exterior surface of each porous particle.

Appellants submit that each of Bergstrom et al., Carlsson et al., Gleason et al. and Velander et al. have been discussed extensively in the background section of the specification. See paragraphs 8, 9, 11 and 12 of the published US application.

Appellants submit that Bergstrom et al. disclose multifunctional porous separation matrix with a layered structure. However, as the Examiner admitted, the structure in Bergstrom et al. does not provide a continuous and smooth ligand gradient. Furthermore, although it is well known that gradients could be designed to

be either stepwise or continuous, Appellants submit that such gradients are generally used in centrifugation or electrophoresis applications. Even though salt or pH or ionic gradients are widely used in separation techniques, they have been used as part of the buffer system. Appellants submit that never before has there been any suggestion that a "continuous and smooth gradient" can be achieved in a separation matrix itself. This kind of gradient gives much better separation of components with similar properties. In comparison, in a step wise gradient such components would end up at the same "step" and are thus not separated from each other.

Appellants submit that although it may be theoretically possible to build a continuous and smooth ligand density gradient by using layer activation like Bergström, one needs to make each activation as a very thin or infinitesimal layer which is hugely impractical. In the case of amine ligands, it is not possible to brominate a second time after the coupling, without destroying the bound amine.

Appellants submit that Carlsson et al. relates to an analytical method for qualitative, semi-quantitative or quantitative determination of at least two analytes in an aqueous sample by thin layer chromatography (TLC). Optionally, the separation zone may have a different ligand density or a gradient of ligand densities along the separation direction (column 6, lines 40-43). Thus, such a density gradient is parallel to the flow during use. In contrast, the claimed matrix includes ligands which provide a chemical gradient that extend between the center and the exterior surface of each porous particle. Furthermore, it appears that Carlsson et al. was mischaracterized by the Examiner in the Advisory Action, since the cited section (i.e., column 7, lines 8-24) does not teach gradients of ligands. Carlsson et al. in the cited section only states that "the ligand density in the detection zone may be varied depending on the mutual concentrations of the different analytes." (column 7, lines 22-24).

Appellants submit that while there are references describing different manners of controlling ligand density on a porous support, they do not teach or suggest a separation matrix as claimed. Gleason et al. disclose a method for controlling/ optimizing ligand density on a polymeric support by introducing a competing quencher molecule. However, there is no teaching for generating a gradient.

Appellants submit that Velander relates to an activated matrix, which can accommodate and optimize the spatial installation of affinity ligands while preventing the immobilization of excess ligand in the outer strata of the hydrogel bead. Velander relates to a uniform functionalization of the inner part of the beads and a lower degree of functionalization of the outer part of the beads compared to the inner part. In the case of cross-linking, Velander aims at a highly and evenly cross-linked inner bead volume and an outer part with a lower degree of cross-linking. The preferred construction is about beads with in principle two discrete and different levels of functionalization or cross-linking. The Examiner states that Velander discloses how varying ligand concentrations may be achieved from the center of a particle to the exterior of the particle. However, Appellants submit that although Velander mentions the possibility of making a gradient of activated sites in column 18 line 36, there is no disclosure as how this can be achieved as an alternative strategy.

In view of the foregoing, Appellants assert that the Examiner's rejection of claims 1, 3-9 and 11-15 cannot be sustained and should be withdrawn.

Appellants respectfully assert that the claims are in allowable form and earnestly solicit the allowance of the claims 1, 3-9 and 11-15.

Conclusion

In view of the foregoing arguments, Appellants respectfully assert that the Examiner's rejections cannot be sustained and should be reversed.

Respectfully submitted,

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I hereby certify that this correspondence is being uploaded to the United States Patent and Trademark Office using the Electronic Filing System on August 5, 2009.

Signature: /Melissa Leck/

Name: Melissa Leck

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CLAIMS APPENDIX

The Rejected Claims

Claim 1 (previously presented): A separation matrix comprising a porous support comprising porous particles; and ligands coupled to the surfaces of said porous support, wherein the ligands provide at least one chemical gradient in the support, which said at least one chemical gradient is a continuous and smooth gradient, further wherein the chemical gradient(s) extend between the center and the exterior surface of each porous particle.

Claim 2 (cancelled)

Claim 3 (previously presented): The separation matrix of claim 1, wherein at least one chemical gradient is a ligand density gradient formed by a changing density of ligands on the support.

Claim 4 (previously presented): The separation matrix of claim 3, wherein two or more chemical gradients are present in the support and at least one gradient is a ligand density gradient.

Claim 5 (previously presented): The separation matrix of claim 3, wherein in the ligand density gradient(s), the ligand concentration increases towards the center of the support.

Claim 6 (previously presented): The separation matrix of claim 3, wherein in the

ligand density gradient(s), the ligand concentration decreases towards the center of the support.

Claim 7 (previously presented): The separation matrix of claim 1, wherein the matrix is a chromatography matrix comprised of a plurality of essentially spherical particles, wherein each particle presents one or more chemical gradient(s) perpendicular to the direction of the liquid flow applied in chromatography.

Claim 8 (previously presented): The separation matrix of claim 1, wherein at least one chemical gradient is the result of varying pKa values of functional groups of the ligands present on the support.

Claim 9 (previously presented): The separation matrix of claim 1, wherein at least one chemical gradient is the result of a varying net charge of the ligands present on the support.

Claim 10 (cancelled)

Claim 11 (previously presented): The separation matrix of claim 1, wherein the ligands present on the porous support provide at least two different functionalities.

Claim 12 (previously presented): The separation matrix of claim 11, wherein said functionalities are selected from the group consisting of cation exchange ligands, anion exchange ligands, hydrophobic interaction chromatography (HIC) ligands, reversed phase chromatography (RPC) ligands, immobilised metal chelating ligands

(IMAC), thiophilic ligands, and affinity ligands.

Claim 13 (previously presented): The separation matrix of claim 11, wherein said at least two different functionalities are present on the same ligand.

Claim 14 (previously presented): The separation matrix of claim 11, wherein the ligands present zwitterionic functionalities.

Claim 15 (previously presented): The separation matrix of claim 11, wherein said at least two different functionalities are present on different ligand kinds, and each such ligand kind provides a separate chemical gradient within the support.

Claims 16-18 (withdrawn)

Claim 19 (cancelled)

Claims 20-52 (withdrawn)

Claim 53 (cancelled)

Claims 54-55 (withdrawn)

EVIDENCE APPENDIX

Appellants hereby append:

1) Bergstrom et al. (US 6,426,315)
2) Carlsson et al. (US 6,528,322)
3) Gleason et al. (US 5,561,097)
4) Velander et al. (US 5,977,345).

These are the evidence relied upon by the Examiner for rejection of appealed claims.

RELATED PROCEEDINGS APPENDIX

There are no other appeals or interferences related to the instant appeal.

US006426315B1

(12) **United States Patent**
Bergstrom et al.

(10) **Patent No.: US 6,426,315 B1**
(45) **Date of Patent: Jul. 30, 2002**

(54) **PROCESS FOR INTRODUCING A FUNCTIONALITY**

(75) Inventors: **Jan Bergstrom**, Balinge; **Rolf Berglund**; **Lennart Soderberg**, both of Uppsala, all of (SE)

(73) Assignee: **Amersham Pharmacia Biotech AB**, Uppsala (SE)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/380,105**

(22) PCT Filed: **Mar. 4, 1998**

(86) PCT No.: **PCT/SE98/00386**

§ 371 (c)(1),
(2), (4) Date: **Mar. 14, 2000**

(87) PCT Pub. No.: **WO98/39364**

PCT Pub. Date: **Sep. 11, 1998**

(30) **Foreign Application Priority Data**

Mar. 4, 1997 (SE) 9700768

(51) **Int. Cl.**[7] **B01J 31/00**; B01J 20/00

(52) **U.S. Cl.** **502/159**; 502/400; 502/401; 502/402; 502/404; 502/439

(58) **Field of Search** 502/159, 400, 502/401, 402, 404, 439

(56) **References Cited**

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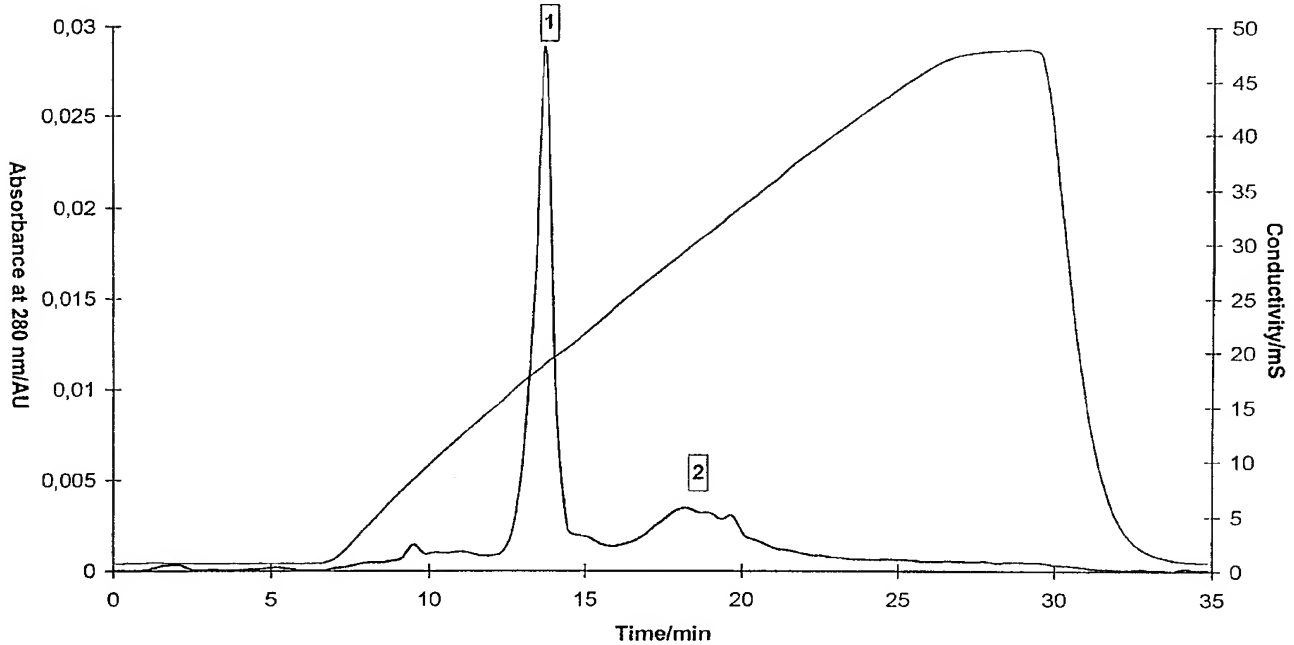
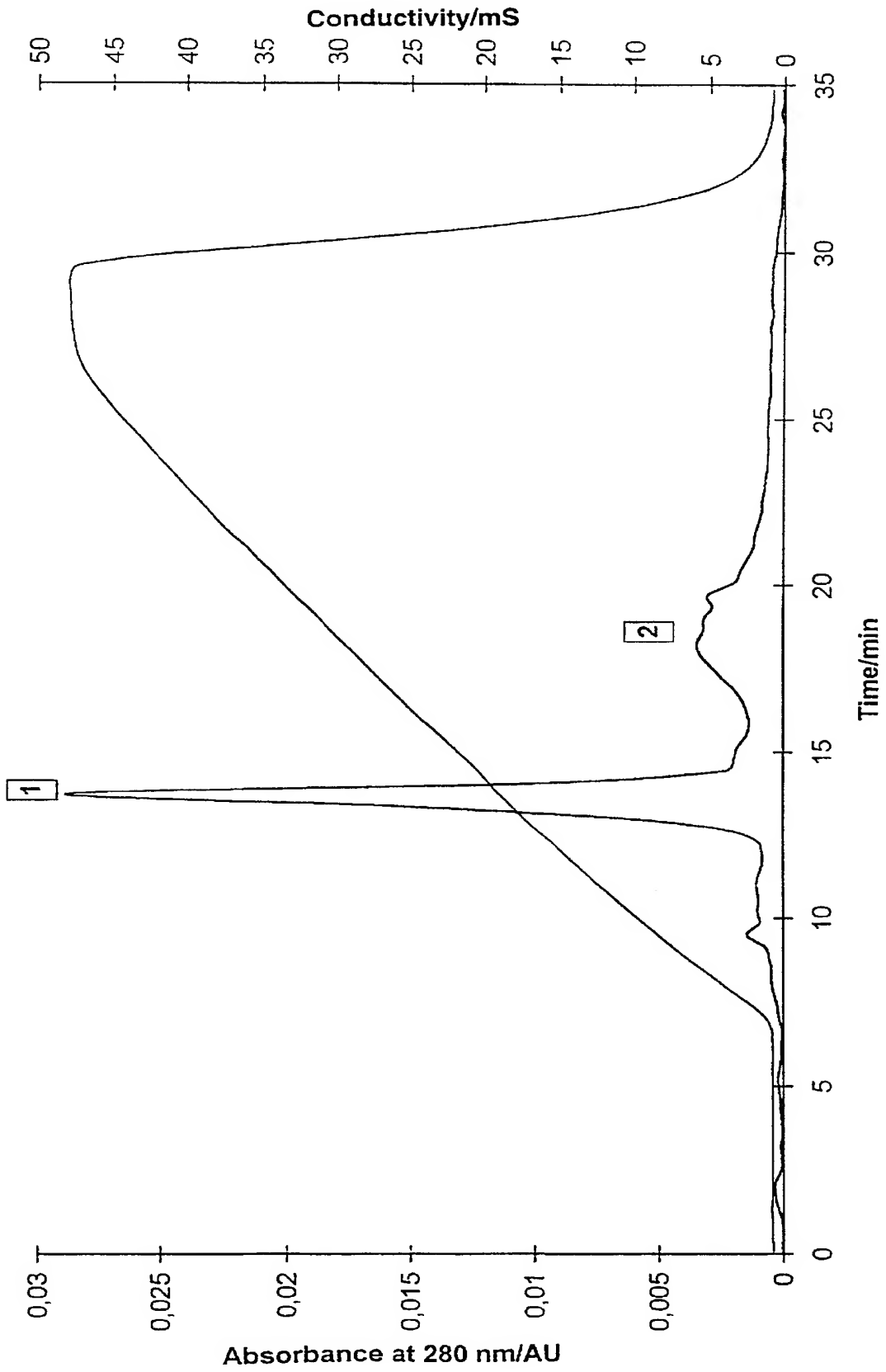
(57) **ABSTRACT**

Process for, in layers of a porous matrix, exhibiting the groups A, introducing a functionality using a reagent I, introducing the functionality by a reaction with the groups A. The characterizing feature is that the matrix is contacted with a functional deficiency of reagent I and that conditions and reagent I are chosen so that the reaction between reagent I and the groups A is more rapid than diffusion of reagent I in the matrix.

9 Claims, 5 Drawing Sheets

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Time/min
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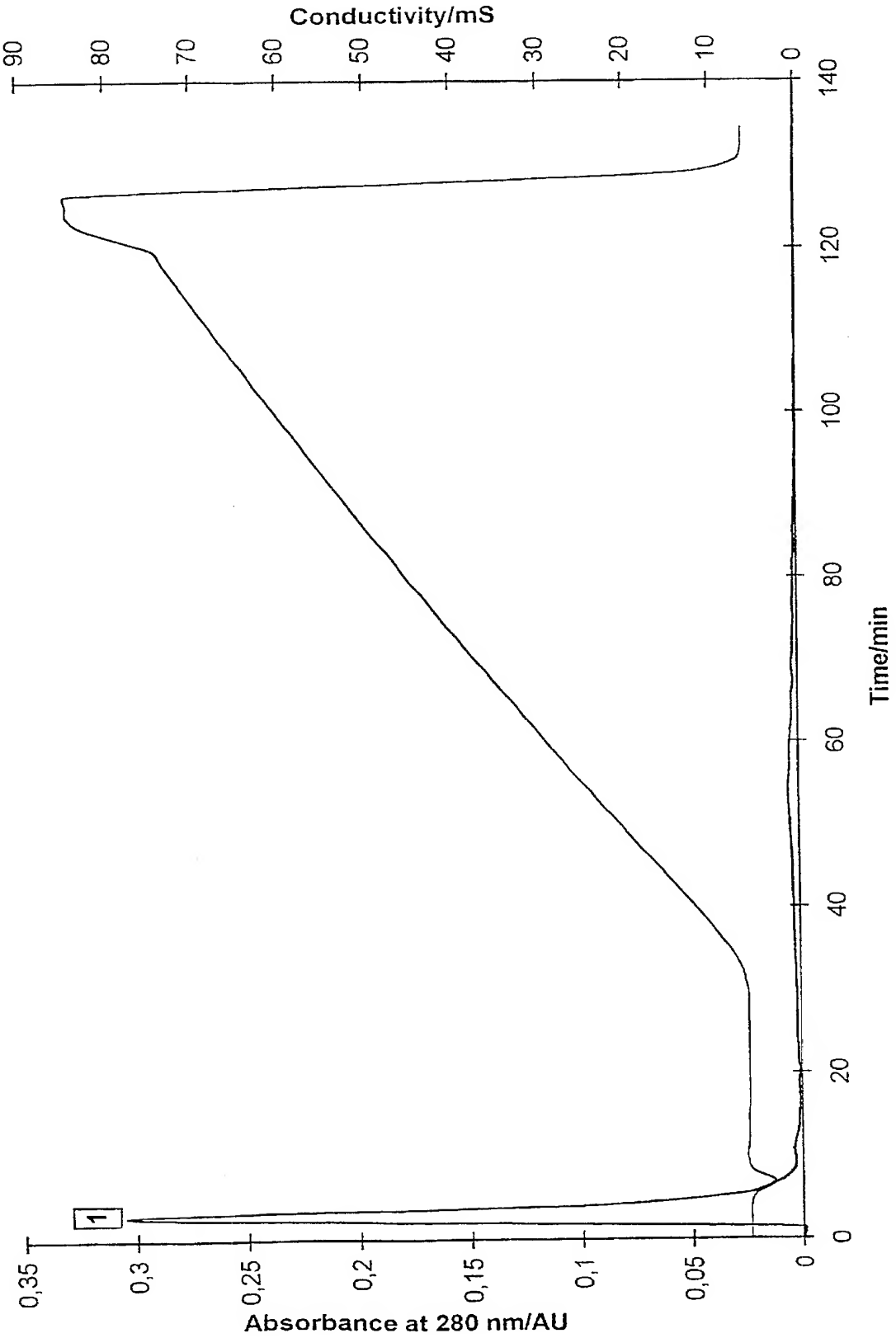
Absorbance at 280 nm/AU

Fig. 1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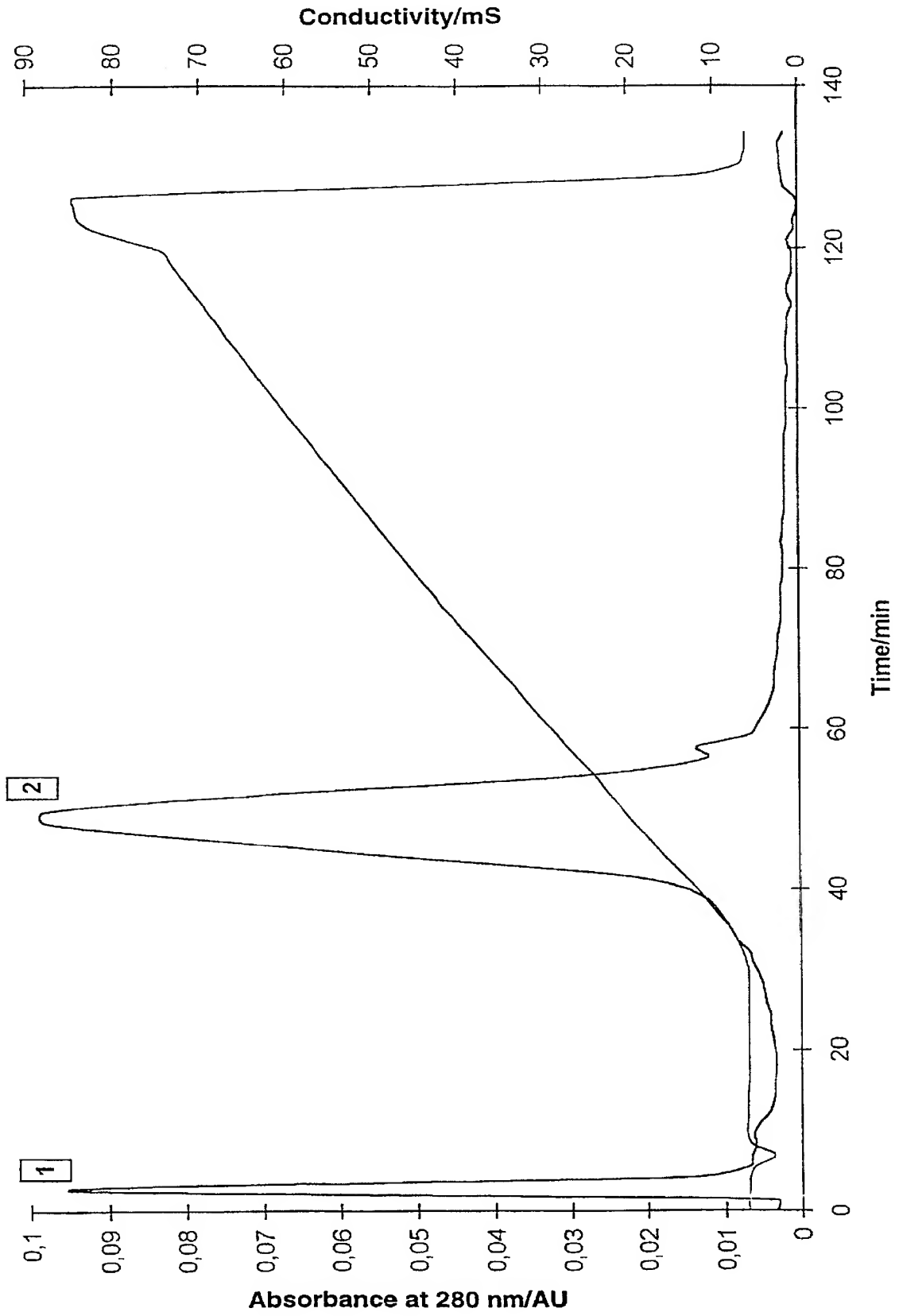
Absorbance at 280 nm/AU

Fig. 2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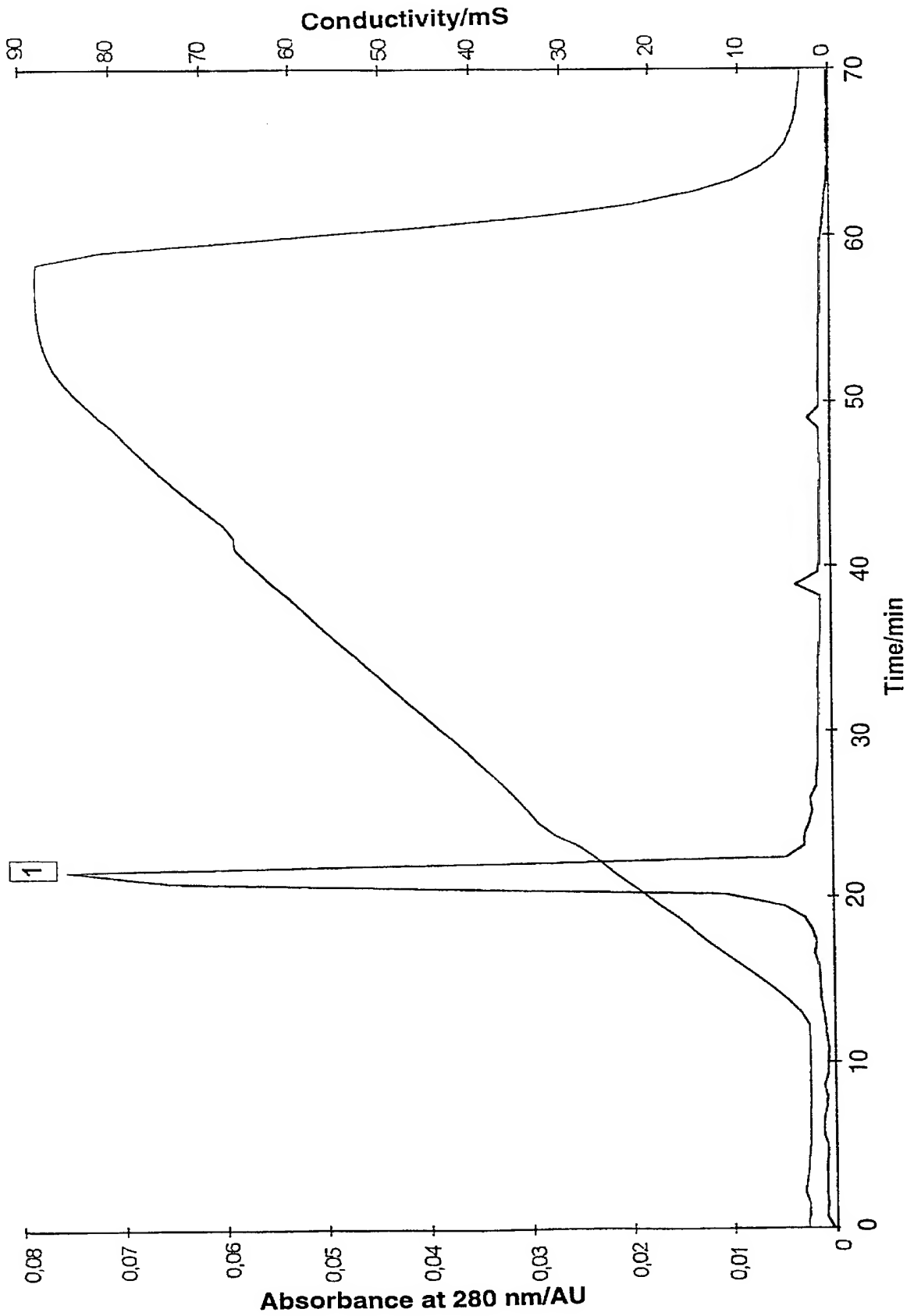
Absorbance at 280 nm/AU

Fig. 3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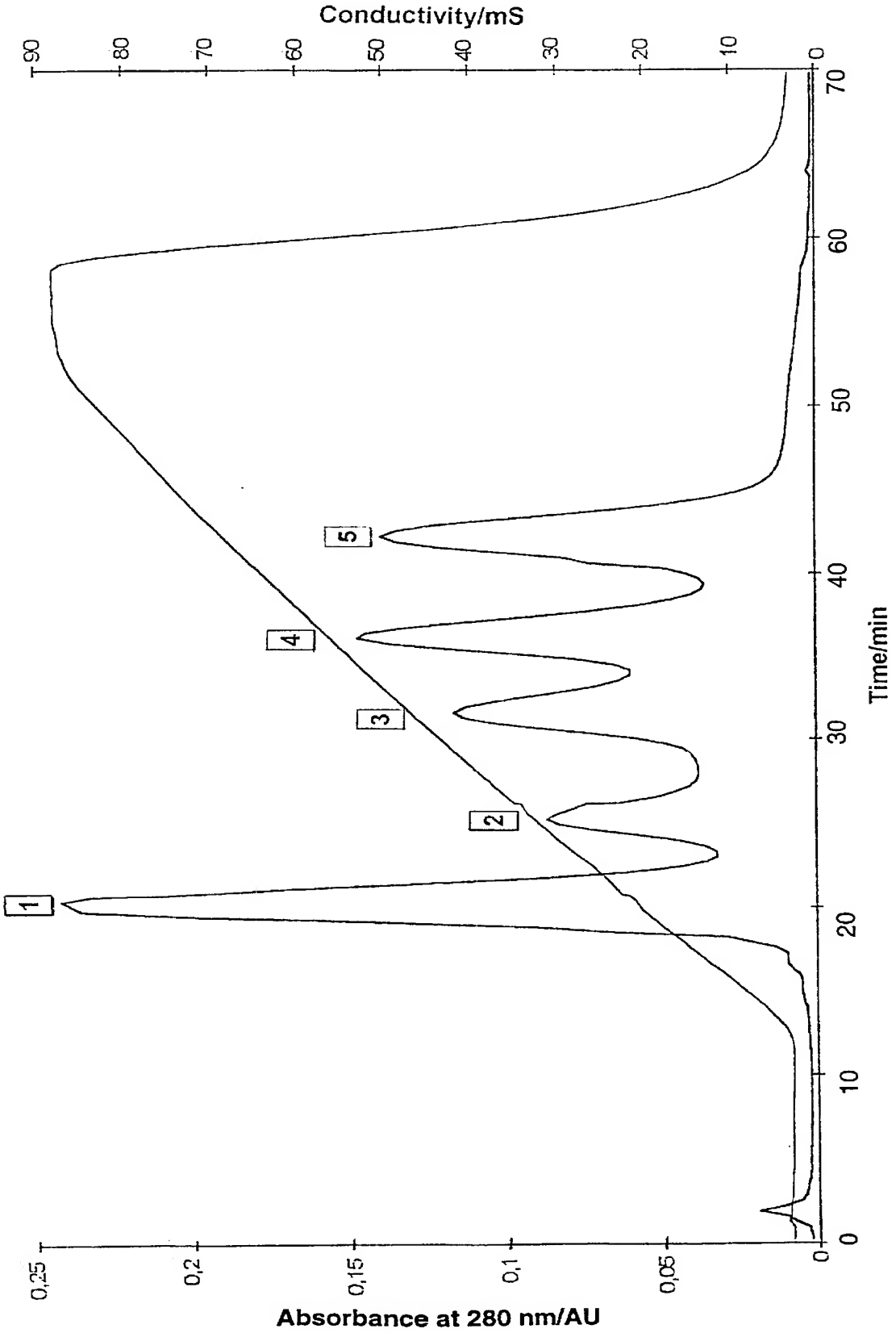
Absorbance at 280 nm/AU

Fig. 4


Conductivity/mS
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Time/min
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Absorbance at 280 nm/AU

Fig. 5

PROCESS FOR INTRODUCING A FUNCTIONALITY

TECHNICAL FIELD AND USE OF THE INVENTION

The invention relates to a process for preparing multifunctional porous matrices. Currently the principal use of the inventive matrices is separation of one or more components from a mixture of components dissolved or dispersed in a liquid. The matrices can also be used as solid phases for: a) the synthesis of oligopeptides and oligonucleotides, b) the analysis and determination methods utilizing affinity reactions, etc. There are also other uses.

Current separations involve that a liquid containing the component(s) is contacted with a matrix, wherein the component(s) to be separated is/are partitioned to the matrix and is/are thereby removed from the remaining components, which are differently partitioned to the matrix including not partitioned at al.

By the expression "partitioned to the matrix" is meant that a component is bound or is otherwise adsorbed on/in the matrix.

By components is meant individual substances/complex structures including whole cells and parts thereof.

TECHNICAL BACKGROUND

Synthesis of Separation Matrices and Associated Problems

To obtain components with sufficiently high purity several separation matrices with different function must often be used in separate steps. This has lead to thoughts of creating multifunctional matrices, which would give a reduction in the number of separation steps. Se e.g. our patent application "Matrices for separation and separation method exploiting said matrices", which has been filed concurrently with this application. We there describe matrices, involving two or more distinct layers which are different concerning separation characteristics.

The synthesis of matrices with given separation characteristics has often involved reacting a base matrix, exhibiting functional groups A, with a reagent I, which via reaction with the groups A gives the matrix a new functionality. A has often been hydroxy, amino (primary, secondary and tertiary), thiol, carboxy (—COOH/—COO—), alkenyl, such as in allyl. halogen etc and corresponding activated (reactive) forms. Since the groups A usually are localized throughout the matrix, earlier known techniques have involved the introduced functionalities have had a similar localization all throughout the matrices. By an introduced functionality is meant either a reactive group or a group contributing to the separation characteristics of the matrix. Reactive groups introduced have then been utilized for creating groups, which contribute to the separation characteristics of the matrix or other characteristics one wishes to utilize in matrix bound form.

According to earlier techniques it has not been possible to introduce layered functionalities in porous matrices. There is a need for new methods.

OBJECT OF THE INVENTION

The principal object of the invention is to enable preparation of porous matrices, which in their interior part exhibit layers with different functions, for separation matrices usually layers with different separating functions (separation layers).

THE INVENTION

The invention is a method for introducing a functionality on a porous matrix according to the methodology mentioned in Technical Background. The invention enables introducing a desired functionality in one or more well defined layers in the matrix. The invention is characterized in

a) that the amount of reagent I, which the matrix is contacted with, is deficient compared to the groups A that exist in advance to reaction with reagent I, and

b) that reagent I and the reaction conditions are selected so that the reaction between reagent I and the group A is rapid compared to transport of reagent I in the matrix (the reaction with group A is rapid compared to the diffusion of reagent I in the matrix).

By deficiency is meant that the amount of reagent I is not sufficient for a reaction with all A groups in the matrix. An inner layer of the matrix will remain unreacted at the same time as a layer with a new functionality is introduced by reagent I. When calculating deficiency, considerations should be made that reagent I can be depleted in side reactions, via vaporization etc.

By rapid reaction between group A and reagent I is also understood that reagent I is adsorbed locally to a group A in a rapid physico-chemical reaction. In this case, reagent I can be stabilized to the matrix by addition of one or more additional reactants promoting local binding to the matrix.

Typical examples group A according to the invention are hydroxy, amino, carboxy (—COOH/—COO—), mercapto, carbon-carbon double bond etc.

Reagent I can be any compound which can bind more rapidly to the matrix than it is diffusing through the matrix, provided that it introduces the intended function. The ratio between rate of diffusion and the rate of reaction with A can be optimized by proper selection of reagent I, taking in consideration that layer functionalisation is promoted by increasing the reactivity of reagent I for group A, and also by increasing the size of reagent I. The reactivity is often influenced by the solvent, pH, temperature etc. Suitable conditions are selected according to conventional practice for each reagent and the type of reaction which is to be performed. Nonpolar and polar organic solvents as well as organic solvents of intermediate polarity and mixtures thereof with each other, and where appropriate also with water, may often be better suited for the process of the invention than pure water.

Reagent I can be a compound which introduces the desired functionality provided the conditions appropriate for this to occur are met (rapid reaction with,the groups compared with transport in the matrix). For the synthesis of separation matrices, in this case reaction of reagent I with A introduces the separating characteristic in a predetermined layer.

Reagent I can also be a so called activating reagent. These are usually utilized for introducing reactive groups that are necessary for further functionalization and use of the matrix according to the introductory part. Activating reagents can be electrophilic, nucleophilic, based on free radicals etc.

On the date of priority we have mostly worked with reagent I in form of electrophilic reagents. Examples are X_2 or XOH (where X is a halogen such as chlorine, bromine, iodine) or any other halogenating reagent which easily gives away a positively or uncharged halogen. Most halogenating reagents, especially X_2 or XOH, give an instantaneous reaction with carbon-carbon double bonds. Carbon-carbon double bonds may result in vicinal dihalides or halohydrins as reactive groups, which easily are converted to reactive epoxy groups.

Usually reactive groups as such do not contribute with any useful separation characteristics. Therefore they are often further reacted with a compound B, which introduces the desired separation characteristic in the layer which has been activated according to the invention. Particular separation characteristics, which can be introduced by use of a compound B are given below under the heading "Introduced Separation Characteristic". Compound B can be selected to introduce reactive groups which in turn can be utilized for introducing separation characteristics via reaction with a compound C. Theoretically longer reaction sequences may be possible to use. Provided that it can be arranged so that compounds B, C etc diffuse slower than they react with previously introduced groups, the invention can also be applied to each of the reaction between compound B. C etc and previously introduced reactive groups.

If required, it can be suitable to introduce protecting groups which later can be removed. For example, if a reagent (reagent I, compound B, C etc) is to be transported through a layer wherein there are groups destroying the reagent.

The degree of cross-linking, the density or the porosity of the matrix is a special kind of functionality. It can be changed layerwise without adding a compound B. For the combination carbon-carbon double bond, as group A, and X_2 or XOH, as reagent I, cross-linking can be achieved in the layer activated according to the invention by a simple change of pH after activation (actually an addition of OH—(=compound B)). Alternatively, in some cases activation can be achieved at a pH which enables cross-linking.

A hydrophilic surface layer is usually obtained, if compound B is water, OH— or some other hydrophilic low molecular nucleophile, which can reacts with an introduced reactive group. If desired, remaining groups A in the inner part of the matrix can then be utilized for functionalization, e.g. according to the invention.

After a separation characteristic has been introduced in a layer, layers with other separation characteristics can be introduced. For example, by renewed activation of the matrix followed by a reaction with a compound B' which introduces separation characteristics other than those obtained with compound B. Additional layers do not necessarily have to be introduced according to the process of the invention. Also in this case there may be a need of introducing protecting groups.

Base Matrices that May Be Used in the Process of the Invention.

Suitable base matrices are often in the form of particles. They should be insoluble but wettable and often also swellable in the liquid medium in which they are to be used. Their inner and outer surfaces can be anything from hydrophilic to hydrophobic. Hydrophilic characteristics are achieved if the matrices on their inner and outer surface exhibit hydrophilic groups such as hydroxy (—OH), amino ($—NH_2$), carboxy (—COOH/—COO—), amido ($—CONH_2$), repeating groups $—OCH_2CH_2—$ and $—OCH_2CH_2CH_2—$, $—OCH_2CH_2(CH_3)—$, etc. Hydrophobic characteristics can be achieved if hydrophobic groups are correspondingly present, e.g. hydrocarbon groups containing 2 or more carbon atoms. For intermediate hydrophilicity/hydrophobicity, the surface of the matrix often exhibits groups of both types.

The matrices are typically built of organic or inorganic polymers which can be of synthetic or biologic origin. Especially so called biopolymers can be mentioned.

Well known hydrophilic organic matrices are polymers exhibiting a number of hydrophilic groups of the types mentioned above. Known hydrophilic polymers are so called polyhydroxy polymers and polyamides, primarily polymers that are insoluble in aqueous media and for instance based on polyvinylic alcohol, poly(hydroxyalkyl methacrylates) and corresponding acrylates, polyacrylic and polymethacrylic amides (e.g. trisacrylic amides and trismethacrylic amides ($tris=(HOCH_2CH_2)_3CNH_3$), polysaccharides such as agarose, dextran, starch, pullulane, and cellulose, optionally cross-linked to make them suitable as separation matrices.

Well known hydrophobic organic matrices are porous forms of styrenedivinyl benzene polymers, poly(alkyl methacrylates), polymers of perfluorinated hydrocarbons (PFC) etc.

Inorganic variants of separation matrices can be based on porous forms of glass, zeolites, silica gel, composite material, zirconium oxide, etc.

Hydrophilic matrices, hydrophobic matrices, inorganic matrices etc. can be given the desired hydrophilicity/hydrophobicity via hydrophilization/hydrophobization.

The most preferred matrices on the date of priority were based on

a) agarose in the form of beads optionally cross-linked and optionally also derivatized with dextran in the pores, e.g. grades marketed under the names of Sepharose® and Superdex®, respectively,

b) cross-linked dextran in the form of beads, e.g. grades marketed under the name of Sephadex®,

c) cellulose, e.g. grades marketed under the name of Sephacel®,

d) cross-linked porous particles of polyacrylamide derivatized with dextran in the pores, e.g. Sephacryl®, and

e) monodispersed and polydispersed porous particles, inter alia, of substantially hydrophobic material, e.g. styrene-divinyl benzene polymer, which have been hydrophilized e.g. grades marketed under the names of MonoBeads® and Source®. These trademarks correspond to products from Amersham Pharmacia Biotech AB, Uppsala, Sweden.

The density of the matrices can be higher, lower or the same as the liquid medium in which they will be used (density for matrix saturated with the liquid medium). Matrices in the form of particles can contain filler agents which determine their density. Se e.g. WO-A-9200799 (KemEnTek/Upfront Chromatography) and WO 9118237 (Pharmacia Biotech AB).

The requirements concerning the porosity (exclusion limit) of the separation matrices are primarily determined by mole weight and shape of the compounds which are to be separated. For the invention, it is also important that the porosity shall permit transport within the matrix of reagent I and often also of compound B.

Interesting exclusion limits are generally in the interval of $3–10^6$ Å. Within the technical field of the applicant, and the future patent owner, it may be especially advantageous with exclusion limits being at least 10 Å.

Introduced Separation Characteristic

The separation characteristics of the matrix are often determined by the groups it carries. Common groups in this context are:

1. ion exchange groups
2. bioaffinity groups
3. hydrophobic groups
4. groups that can be utilized for covalent chromatography
5. sulphur-containing groups e.g. for so called thiophilic interaction,

6. chelate or chelating groups,
7. groups with aromatic systems giving rise to so called π—π-interaction with different compounds,
8. groups giving hydrogen bonds
9. cross-linking groups
10. Hydrophilic groups etc.
11. polymeric groups.

In the context of the invention, it is often arranged so that compound B or B' or the corresponding entities in subsequent reactions exhibitany of the groups **1–11**. These groups can also be created as a consequence of the reaction between compound B and reactive groups being introduced. in a layer according to the invention in an earlier step. The latter is especially true for smaller groups of type **1** or any of the types **3–9**. In some cases, the groups can also be introduced directly with reagent I.

Ion exchanging groups can be anion exchanging, such as primary, secondary, tertiary, quaternary ammonium group, sulphonium group etc, or cation exchanging, such as carboxylate (—COO—), phosphonate or phosphate (—PO_3^2— and —OPO_3^2—, respectively), sulphonate or sulphate (—SO_3— and —OSO_3—, respectively) etc. In the groups —COO—, —PO_3^2—, —OPO_3^2—, —SO_3— and —OSO_3— the free valence binds directly to a carbon atom.

Well known bioaffinity groups are single members of the pairs a) antigen/hapten and antibody (including antigen or hapten binding fragment thereof), b) nucleic acid and its complementary counterpart, c) lectin and carbonhydrate structure, d) IgG binding protein and protein exhibiting the part of IgG that binds to such a protein, e) sense and antisense based affinitysystems etc. Bioaffinity groups also include groups originating from synthetically prepared organic molecules and which "mimic" the affinity for naturally occurring biospecific affinity, so called "mimetics".

Hydrophobic groups are often hydrocarbon groups containing a few or no oxygen, nitrogen or sulphur atoms. Typical examples of hydrophobic groups are straight, branched and cyclic saturated, unsaturated or aromatic hydrocarbon groups.

Among groups which can be utilized for covalent chromatography can be mentioned disulphide groups, mainly reactive disulphide groups (—S—S—R^1) and free thiol groups (—SH). An example of R_1 is 2-pyridyl. For further examples of R^1 see e.g. U.S. Pat. No. 4,647,655 (Pharmacia AB).

Among sulphur containing groups which can be utilized for thiophilic interaction there can be mentioned groups being essentially hydrophobic but in which there is one or more thioether structures. See e.g. Oscarsson & Porath WO-A-9533557; Porath EP-A-165912; and Porath EP-A-168363.

Hydrogen binding groups have previously been utilized (Belew, Berglund, Bergström, Söbderberg, SE 9600590-5 (=WO 97 29825) (incorporated by reference). This type of groups often exhibits a weak anion exchanging ammonium group (primary, secondary or tertiary) with a hydroxy group at a distance of 2 or 3 carbon atoms from the ammonium nitrogen.

Cross-linking groups can be introduced directly by use of reagent I. See above. Alternatively a compound B can be utilized, which is capable of binding two or more reactive groups simultaneously.

Hydrophilic groups according to the invention are mainly single hydroxy and lower hydroxyalkyl with one or more hydroxyl groups or groups containing repeating groups —CH_2CH_2O—. The groups often are low molecular, e.g. less than 25 carbon atoms.

Polymeric groups with or without ligands can give gel filtration characteristics. The polymers can be cross-linked.

Suitable groups are typically coupled to the matrix via a bridge which can be of varying structure according to known techniques. The bridge structure can be polymeric, e.g. a hydrophilic or a hydrophobic polymer, having one or more of the groups **1–11** according to the above on each bridge. Common bridge names have been tentacles, "extender", fluff, "linker", "spacer" etc. Hydrophobic bridges are mainly suited for hydrophobic liquid media and often have better availability and capacity for introduced groups **1–11**. The corresponding is true for hydrophilic bridges in combination with hydrophilic liquid media. Examples of hydrophilic polymer bridges are polysaccharides, such as dextran, and other water soluble polyhydroxy polymers.

Further Aspects of the Invention

One aspect of the invention is matrices which can be prepared according to the invention. These matrices contain one or more layers having different functionality. The substitution degree for at least one ligand from the groups **1–11** in one layer is often different from the substitution degree for the same ligand in another layer. In many embodiments of the matrices of the invention, the substitution degree of a ligand in the surface layer is zero or close to zero, while at the same time the same ligand is present in an inner layer. Also the reversed can be true.

A further aspect of the invention is the use of the matrices for separation according to the introductory part.

Separation, depending on choice of matrix and groups introduced (see above), can be designed as affinity chromatography or as chromatography based on size and shape of the compounds which are to be separated (gel filtration), or as corresponding batchwise procedures. Packed bed as well as stabilized fluidized/expanded bed can be utilized. Examples of affinity chromatography are ion exchange chromatography (anion exchange, cation exchange), bioaffinity chromatography, hydrophobic interaction chromatography (HIC), covalent chromatography, thiophilic chromatography, chelate based chromatography, chromatography based on π—π-interaction etc. In principle, conditions and protocols are chosen in accordance with previous knowledge for the respective type of separation procedure.

The separation can be performed from mixtures containing similar or very different components, everything from single small molecules up to individual components that are complexly attached to each other, such as in particulate aggregates, bioaffinity complexes, animal and plant cells and parts there of, microorganisms and parts thereof etc. Interesting substances are, inter alia, nucleic acid including oligonucleotides, proteins including peptides, lipids and other organic and inorganic compounds.

Separation can involve that the component of interest: a) is partitioned to the matrix while undesired substances remain in the liquid medium or b) remains in the liquid medium while undesired substances are partitioned to the matrix.

The invention will now be presented with a number of non-limiting examples. The invention is defined in the enclosed patent claims constituting a part of the specification.

EXPERIMENTAL PART

Example 1

Preparation of Q Sepharose 4 Fast Flow Ion Exchanger with a Lock in the Outer Layer of the Bead

A. Preparation of cross-linked allylated agarose in particle form (allylated Sepharose 4 Fast Flow). Sepharose 4 Fast

Flow was from Amersham Pharmacia Biotech AB, Uppsala, Sweden. The matrix consists of cross-linked agarose, average particle size 90 μm, prepared by a reaction between epichlorohydrin and agarose in the presence of NaOH according to Porath et al (J. Chromatog. 60 (1971) 167–77 and U.S. Pat. No. 3,959,251). Allylation is achieved by reacting the finished particle with allylglycidyl ether with NaOH as a base to an allyl level (CH_2=$CHCH_2OCH_2CHOHCH_2$—) of 0.26 mmole/mL gel.

B. Dissolving of dextran with a weighed average molecular weight of 500 000. Dextran T500 is commercially available from Amersham Pharmacia Biotech AB and consists of hydrolyzed and fractioned raw dextran from Leuconostoc mesenteroides. 12.8 g of Dextran T500 is dissolved in 39.0 mL distilled water in a 250 mL three necked flask with slow stirring.

C. Partial bromination of cross-linked allylated agarose. 30 mL drained allylated agarose prepared according to step A, 30 mL distilled water and 0.64 g anhydrous NaOAc are added to a 100 mL reaction vessel. Subsequently bromination is achieved by loading of 0.25 mL bromine with vigorous stirring. On calculation of the amount of bromine, it has been taken into consideration that a part of the volatile bromine will be evaporated. The reaction is continued for a few minutes until the mixture is purely white. After bromination, the gel is washed on a glass filter with distilled water.

D. Coupling of Dextran T500. The gel from step C is aspirated dry and transferred to the reaction vessel containing dissolved Dextran T500 from step B with careful stirring. The mixture is allowed to equilibrate for 1 hour. Subsequently, the reaction is started by addition of 2.98 g NaOH and 0.12 g $NaBH_4$ dissolved in 27.2 mL distilled H_2O. The temperature is set to 35° C. and the reaction is allowed to continue over night (e.g. 16 h) with careful stirring. The stirring is stopped and the reaction mixture is filtered on a glass filter. After the most of Dextran T500 has been washed away with distilled water, neutralisation is performed with a few mL of concentrated HOAC directly in the filter funnel to pH <7, preferably 5–6, and then the gel is washed again with distilled water. After coupling, the remaining allyl level was 0.19 mmole/mL.

E. Bromination of remaining allyl groups. 20 mL drained allylated agarose modified with a layer of Dextran T500 in the outer part of the beads, prepared according to step D, 4.53 mL distilled water and 0.8 g anhydrous NAOAc and 0.4 mL bromine are added with vigorous stirring to a 100 mL three-necked flask. Stirring is continued until the yellow colouring/excess of bromine is eliminated.

F. Introduction of ion exchanging groups via remaining allyl groups. The introduction of anion exchanging quaternary amine groups $(CH_3)_3N+CH_2CHOHCH_2$— is continued directly in the same three necked flask as in E. 9.9 g of 65% trimethyl ammoniumchloride (TMAC) is loaded into the flask. Stirring for 10 minutes and then loading of 3.0 g of NaOH dissolved in 3.0 g distilled water. On loading of this 50% NaOH solution the mixture becomes pale yellow. Immediately thereafter 0.06 g of $NaBH_4$ is loaded thereto. The temperature is set to 24° C. and stirring is initiated. The reaction is continued over night (e.g. 16 h). The reaction is stopped by neutralization directly in the flask to pH <7 with a few mL of concentrated HOAc, preferably to pH 5–6, and then the gel is washed repeatedly by distilled water and with a few gel volumes of 1.0 M NaCl. Eventually, the gel is washed again repeatedly with distilled water. The capacity of ion exchange was determined by silver chloride titration to 0.132 mmole/mL.

G. Microscopic evaluation of Q Sepharose 4 Fast Flow Ion Exchanger with a lock in the Outer Layer of the Bead. All microscopic evaluations were conducted in a microscope with variable setting of phase contrast at a magnification of 200. The starting matrix Sepharose 4 Fast Flow of example 1 and the and finished Q Sepharose 4 Fast Flow ion exchanger with a lock of example 1 were stained with haematoxylin for comparison. In the latter case, the lock could be seen clearly in the outer layer of the beads in the microscopic photo taken. To be able to observe optically that the lock beads are working, finished Q Sepharose 4 Fast Flow lock ion exchanger was incubated for a few minutes with bovine CO-haemoglobin at pH 8.2; 0.020 M Tris-HCl buffer. The excess of CO-haemoglobin was washed away with the same buffer. The beads were then observed in a microscope without any previous additional staining. On a the microscopic photo the red CO-haemoglobin in the inner part of the beads could be seen but nothing of the CO-haemoglobin could be seen in the Dextran T500 lock layer of the beads.

Example 2

Preparation of Q Sepharose 6 Fast Flow Ion Exchanger with a Lock in the Outer Layer of the Bead

A. Preparation of cross-linked allylated agarose in particulate form (allylated Sepharose 6 Fast Flow). Sepharose 6 Fast Flow was from Amersham Pharmacia Biotech AB, Uppsala, Sweden. The matrix consists of cross-linked agarose, average particle size 90 μm, prepared by reaction of epichlorohydrin and agarose in the presence of NaOH according to Porath et al (J. Chromatog. 60 (1971) 167–77 and U.S. Pat. No. 3,959,251). Allylation is performed in such a way that the finished particle is reacted with allyglycidyl ether with NaOH as a base to a allyl level (CH_2=$CHCH_2OCH_2CHOHCH_2$—) of 0.27 mmole/mL gel.

B. Dissolving of Dextran with a weighed average molecular weight of 500 000. Dextran T500 is commercially available from Amersham Pharmacia Biotech AB and consists of hydrolyzed and fractionalized raw dextran from Leuconostoc mesenteroides. 57.7 g of Dextran T500 is dissolved in 157 mL distilled water in a 500 mL three necked flask with slow stirring.

C. Partial bromination of cross-linked allylated Agarose. 90 mL drained allylated agarose prepared according to step A, 90 mL distilled water and 1.92 g of anhydrous NaOAc are added to a 500 mL three-necked flask. Subsequently bromination is achieved by loading of 0.69 mL bromine with vigorous stirring. On calculation of the amount of bromine, it has been taken into consideration that a part of the volatile bromine will be evaporated. The reaction is continued for a few minutes until the mixture is purely white. After bromination the gel is washed on a glass filter with distilled water.

D. Coupling of Dextran T500. The gel from step C is aspirated dry and transferred to the reaction vessel with dissolved Dextran T500 from step B with careful stirring. The mixture is allowed to equilibrate for 1 hour. Subsequently the reaction is started by addition of 10.39 g NaOH and 0.36 g $NaBH_4$ dissolved in 41.6 mL distilled H_2O. The temperature is set to 35° C. and the reaction is allowed to continue over night (e.g. 16 h) with careful stirring. The stirring is stopped and the reaction mixture is filtered on a glass filter. After washing away of most of the Dextran T500 with distilled water, neutralisation is performed with a few mL of concentrated HOAc directly in the filter funnel to pH

<7, preferably 5–6, and then the gel is washed again with distilled water. After coupling the remaining allyl level was 0.18 mmole/mL.

E. Bromination of remaining allyl groups. 40 mL drained allylated agarose with a layer of Dextran T500 in the outer part of the beads, prepared according to step D, 9.06 mL distilled water and 1.6 g anhydrous NaOAc and 1.0 mL bromine are added with vigorous stirring to a 250 mL three-necked flask. Stirring is continued until the yellow colouring/excess of bromine is eliminated.

F. Introduction of ion exchanging groups via remaining allyl groups. The introduction of an ion exchanging quaternary amine groups $(CH_3)_3N+CH_2CHOHCH_2$— is continued directly in the same three-necked flask as in E. 19.9 g of 65% trimethyl ammoniumchloride (TMAC) is loaded into the flask. Stirring for 10 minutes and then loading of 6.0 g of NaOH dissolved in 6.0 g distilled water. On loading of this 50% NaOH solution the mixture becomes pale yellow. Immediately thereafter 0.12 g of $NaBH_4$ is loaded. The temperature is set to 24° C. and the stirring rate to 130 RPM and the reaction is continued over night (e.g. 16 h). The reaction is stopped by neutralization directly in the flask to pH <7 with a few mL of concentrated HOAc, preferably to pH 5–6, and then the gel is washed repeatedly by distilled water and with a few gel volumes of 1.0 M NaCl. Eventually the gel is repeatedly washed again with distilled water. The capacity of ion exchange was determined by silver chloride titration to 0.142 mmole/mL.

G. Chromatographic evaluation of Dextran T500 Q Sepharose 6FF. The effect of the outer dextran layer was tested by chromatography utilizing gradient runs on HR5/5 columns (Amersham Pharmacia Biotech AB) packed with about 1 mL ion exchanger. A large protein thyroglobulin 660 kD, and a small α-lactalbumin 14.4 kD were used as samples at pH 8.2, at which both are negatively charged. FIG. **1** shows the separation of both proteins in a gradient run on a conventional strong anion exchanger, Q-Sepharose HP, without size based restriction of the availability to the positively charged Q ligands. Here α-lactalbumin (peak 1) elutes before thyroglobulin (peak 2). FIG. **2** shows the result from a gradient run with thyroglobulin on Dextran T500 Q Sepharose 6FF ion exchanger according to this example. This large protein migrates right through the column without being adsorbed. The smaller α-lactalbumin which was run in a similar way is adsorbed and eluted in the gradient (peak 2). See FIG. **3**.

Example 3

Synthesis of Cat-anion Exchanger by Use of Layer Activation

A. Synthesis. 5 ml of allylated Sepharose HP with an allyl level of about 0.2 mmole/mL, prepared analogously with the procedure in examples 1 and 2 by reacting Sepharose HP with allylglycidyl ether in water with sodium hydroxide as a base, was suspended in 20 mL distilled water in a glass reaction vessel and was brominated with vigorous stirring with 0.3 mmole elementary bromine. The partially brominated gel was then reacted with 25 mL of 0.1 M sodium hydroxide solution saturated with sodium sulphite. Reaction at 40° C. over night. The reaction was stopped by neutralization with acetic acid and subsequent washing in a glass filter funnel with about 100 mL distilled water. Remaining allylic groups on the partially functionalized matrix were brominated in 20 mL distilled water with an excess of bromine by dropwise loading of elementary bromine until a remaining yellow colouring of the gel suspension was obtained. The brominated partially functionalized matrix was then washed on a glass filter funnel with distilled water and loaded in 25 mL of a 50% solution of bis-(3-amino propyl)amine. Reaction at room temperature with stirring over night. The reaction was stopped by neutralization with 50% hydrochloric acid and subsequent washing in a glass filter funnel with about 100 mL distilled water.

B. Chromatographic evaluation of layered cat-anion exchanger. About 1 mL of cat-anion exchanger prepared according to specification above was packed in a HR5/5 column (Amersham Pharmacia Biotech AB), was connected to a FPLC system and equilibrated with a pH 6.0 buffer. The protein samples were adsorbed and then eluted by use of a salt gradient also at pH 6.0.

Sample A. Lysozyme, a protein which is positively charged at pH 6.0, was run according to above. See chromatogram in FIG. **4**, which shows that lysozyme can be bound to the negative sulphonate ligands of the matrix and then be eluted.

Sample B. Transferrin, ovalbumin and β-lactoglobulin, proteins which are negatively charged at pH 6.0, were run according to above. See chromatogram in FIG. **5**, which shows that transferrin, ovalbumin and β-lactoglobulin can be bound to the positively charged amine ligands of the matrix and then be eluted.

The result from chromatographv tests A and B shows that there is regions/layers in the particles, which only contain positively charged groups and regions/layers with only negatively charged groups.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Chromatogram from the 50 μl sample mixture run containing 0.5 mg/mL α-lactalbumin (peak 1) and 0.5 mg/mL thyroglobulin (peak 2) on a HR 5/5 column (Amersham Pharmacia Biotech) containing Q Sepharose HP, bed height 5.9 cm.

Buffer A: 20 mM Tris-HCl pH8.2.

Buffer B: 20 mM Tris-HCl+0.5 M NaCl

Flow rate: 1 mL/min

FIG. **2**. Chromatogram from the 200 μl sample run containing 0.25 mg/mL α-lactalbumin on a HR 5/5 column (Amersham Pharmacia Biotech) containing Dextran T500 Q Sepharose 6FF according to example 2, bed height 6.5 cm.

Buffer A: 20 mM Tris-HCl, 50 mM NaCl pH 8.2.

Buffer B: 20 mM Tris-HCl+1 NaCl

Flow rate: 0.2 mL/min

FIG. **3**. Chromatogram from the 200 μl sample run containing 1 mg/mL thyroglobulin on a HR 5/5 column (Amersham Pharmacia Biotech) containing Dextran T500 Q Sepharose 6FF according to example 2, bed height 6.5 cm.

Buffer A: 20 mM Tris-HCl, 50 mM NaCl pH 8.2.

Buffer B: 20 mM Tris-HCl+1 NaCl

Flow rate: 0.2 mL/min

FIG. **4**. Chromatogram from a 50 μl sample run containing 1 mg/mL lysozyme on a HR 5/5 column (Amersham Pharmacia Biotech) containing cat-anion exchanger according to example 3, bed height 4.2 cm.

Buffer A: 20 mM piperazine —HCl pH 6.0

Buffer B: 20 mM piperazine —HCl+1 NaCl

Flow rate: 0.5 mL/min

FIG. **5**. Chromatogram from a 50 μl sample run containing 0.1 mg/mL transferrin (peak 1), 0.2 mg/mL ovalbumin (peaks 2 and 3) and 0.2 mg/mL β-lactoglobulin (peak 4 cat-anion exchanger according to example 3, bed height 4.2 cm.

Buffer A: 20 mM piperazine —HCl ph 6.0

Buffer B: 20 mm piperazine —HCl+1 NaCl

Flow rate: 0.5 mL/min

What is claimed is:

1. In a process for introducing a functionality in layers of a porous matrix containing groups A, for use of a reagent I by reaction of said functionality with the said groups A, the improvement comprising contacting the matrix with a deficiency of reagent I, under conditions such that the reaction of reagent I and the groups A is more rapid than the diffusion of reagent I in the matrix.

2. A process according to claim **1**, characterized in that A is a carbon-carbon double bond and that reagent I is a halogenating reagent.

3. A process according claim **1**, characterized in that reagent I is X_2 or XOH, wherein X is a halogen selected from chlorine, bromine and iodine.

4. A process according to claim **1**, characterized in that the functionality which is being introduced is a reactive group that in a subsequent step is reacted with a compound B introducing a desired separation characteristic to the matrix.

5. A process according to claim **1**, characterized in that the matrix is hydrophilic and exhibits hydrophilic groups on its inner and outer surfaces.

6. A process according to claim **1**, characterized in that the matrix is built of a polyhydroxy polymer.

7. A process according to claim **1**, characterized in that the reaction of the groups A and reagent I is performed in an aqueous medium.

8. A process according to claim **4**, characterized in that the desired separation characteristic introduced by compound B is affinity chromatography.

9. A process according to claim **4**, characterized in that the desired separation characteristic introduced by compound B is gel filtration.

* * * * *

US006528322B1

(12) **United States Patent**
Carlsson et al.

(10) **Patent No.: US 6,528,322 B1**
(45) **Date of Patent: Mar. 4, 2003**

(54) **ANALYTICAL METHOD AND APPARATUS**

(75) Inventors: **Jan Carlsson**, Uppsala (SE); **Maria Lönnberg**, Knivsta (SE)

(73) Assignee: **Pharmacia AB**, Stockholm (SE)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 153 days.

(21) Appl. No.: **09/633,110**

(22) Filed: **Aug. 4, 2000**

Related U.S. Application Data

(60) Provisional application No. 60/148,566, filed on Aug. 13, 1999.

(30) **Foreign Application Priority Data**

Aug. 6, 1999 (SE) 9902855

(51) **Int. Cl.**[7] **G01N 33/543**

(52) **U.S. Cl.** **436/514**; 436/518; 436/528; 436/530; 436/161; 436/102; 435/7.1; 435/287.9; 435/288.3; 204/400

(58) **Field of Search** 435/7.1, 287.9, 435/288.3; 436/518, 514, 528, 530, 161, 162; 204/400

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Pristoupil, T. I., Chromatog. Rev., vol. 12 (1970) pp. 109–125.

Primary Examiner—Bao-Thuy L. Nguyen

(74) *Attorney, Agent, or Firm*—Birch, Stewart, Kolasch & Birch, LLP

(57) **ABSTRACT**

The invention relates to a method for qualitative, semi-quantitative or quantitative determination of at least two analytes in an aqueous sample containing or suspected of containing the analytes, which method comprises the steps of:

(i) providing a flow matrix comprising a separation zone extending in a first dimension thereof, and a detection zone extending in the first dimension in a spaced parallel relationship with the separation zone, the detection zone comprising an immobilized reagent capable of capturing the analytes through biospecific interaction therewith,

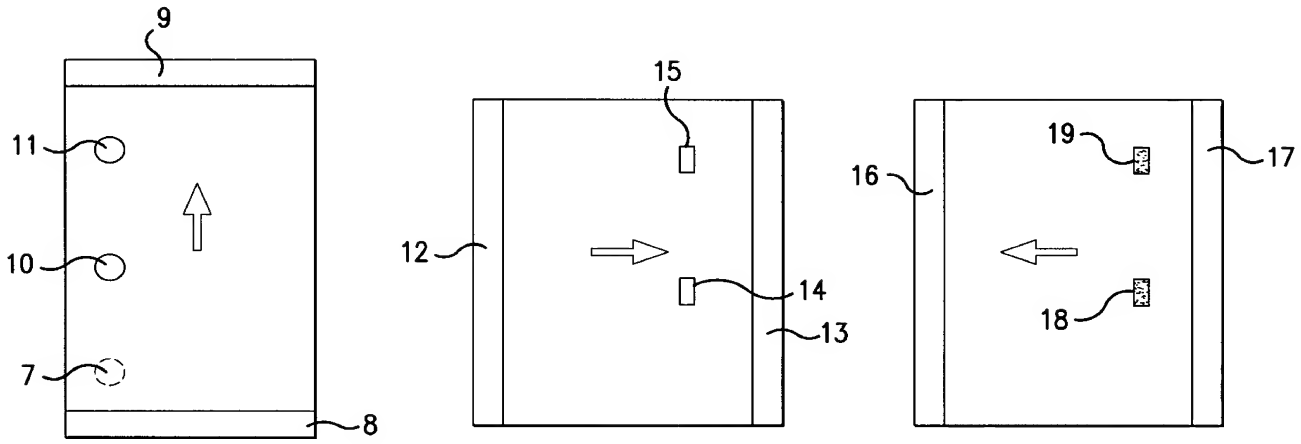
(ii) applying the sample to the flow matrix at or upstream of the separation zone,

(iii) initiating a first essentially aqueous fluid flow in the flow matrix along the separation zone in the first dimension to transport the analytes through the separation zone to be separated therein,

(iv) interrupting the first fluid flow and initiating a second essentially aqueous fluid flow in a second dimension of the flow matrix substantially transverse to the first dimension towards the detection zone to transport the separated analytes to the detection zone to be captured therein by the immobilized reagent, and

(vi) determining the analytes in the detection zone. The invention also relates to an apparatus for carrying out the method.

33 Claims, 3 Drawing Sheets



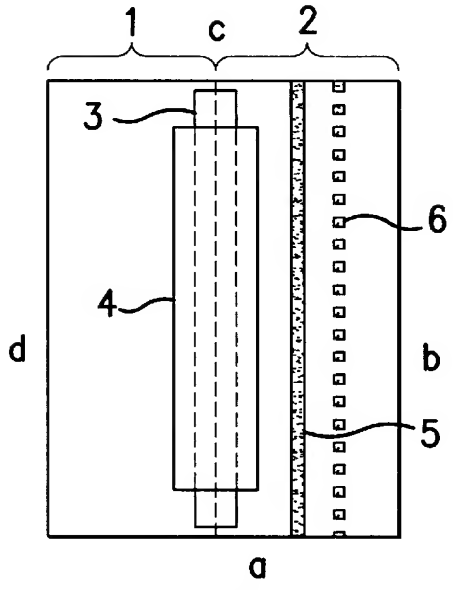


FIG.1

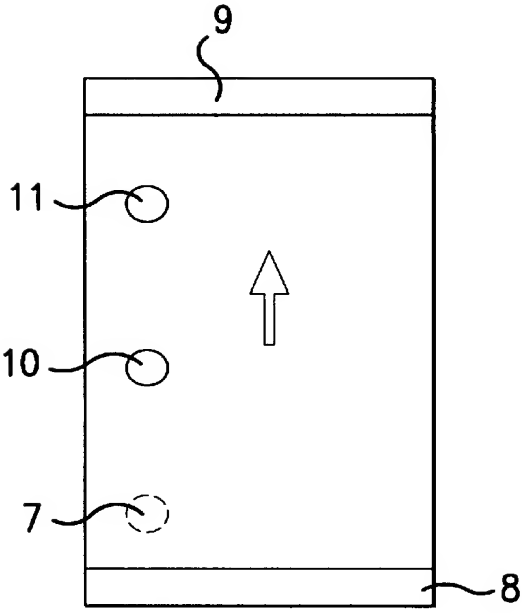


FIG.2A

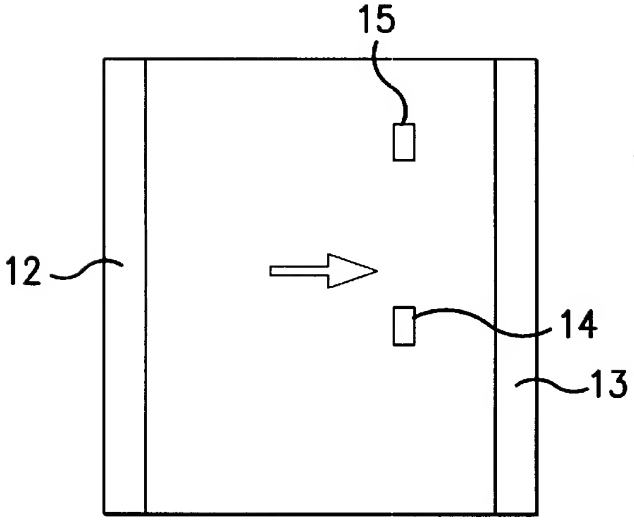


FIG.2B

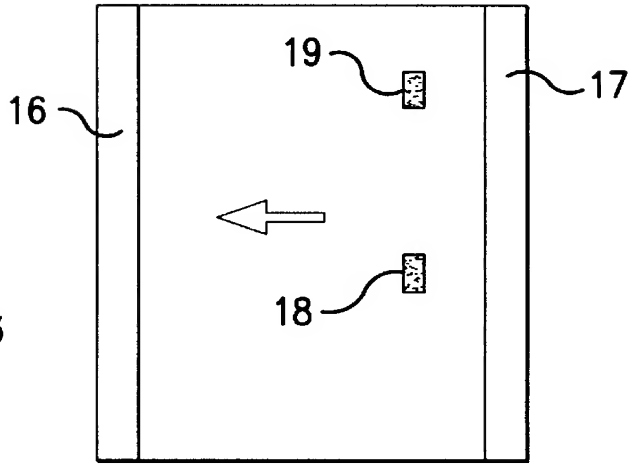


FIG.2C

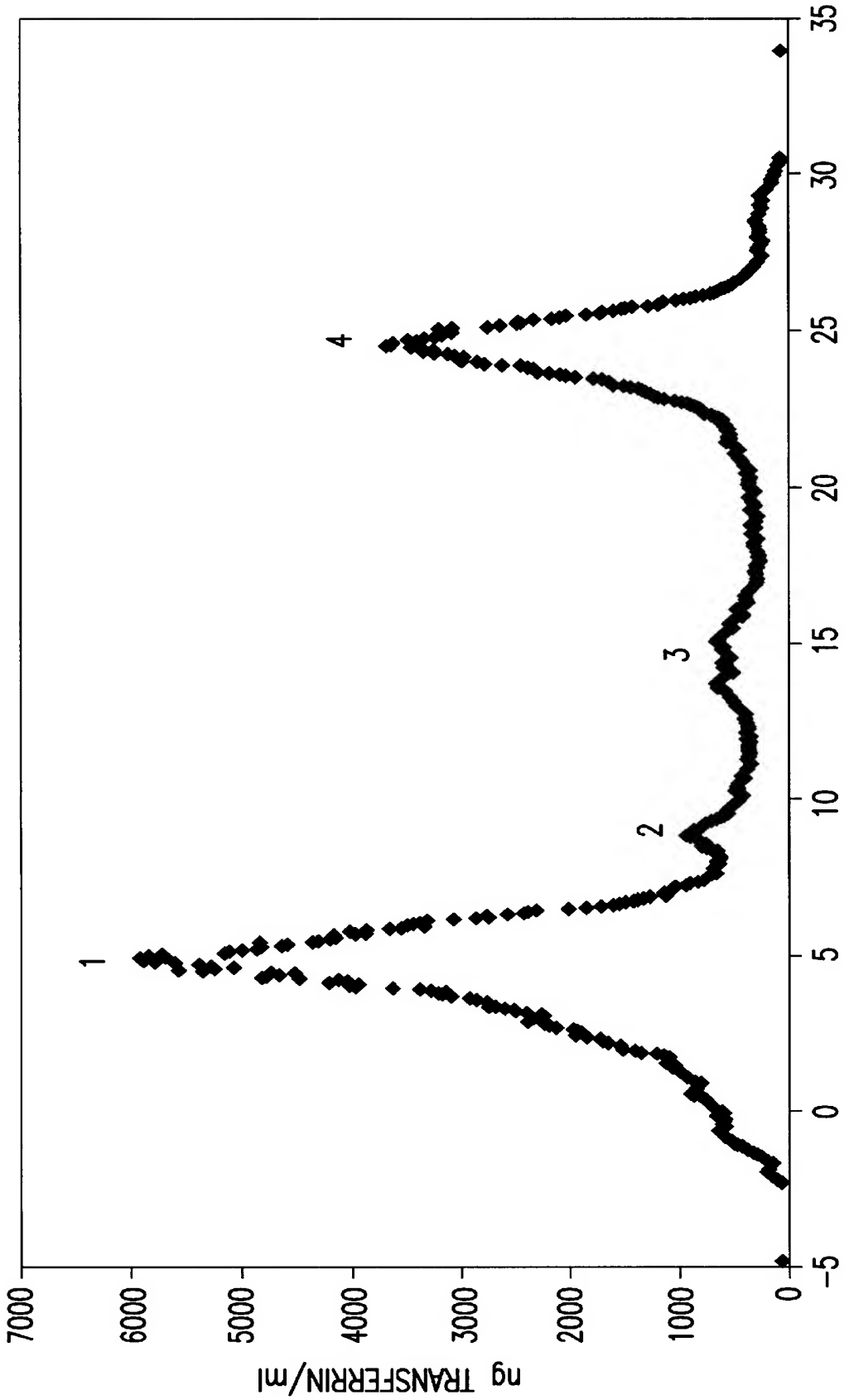

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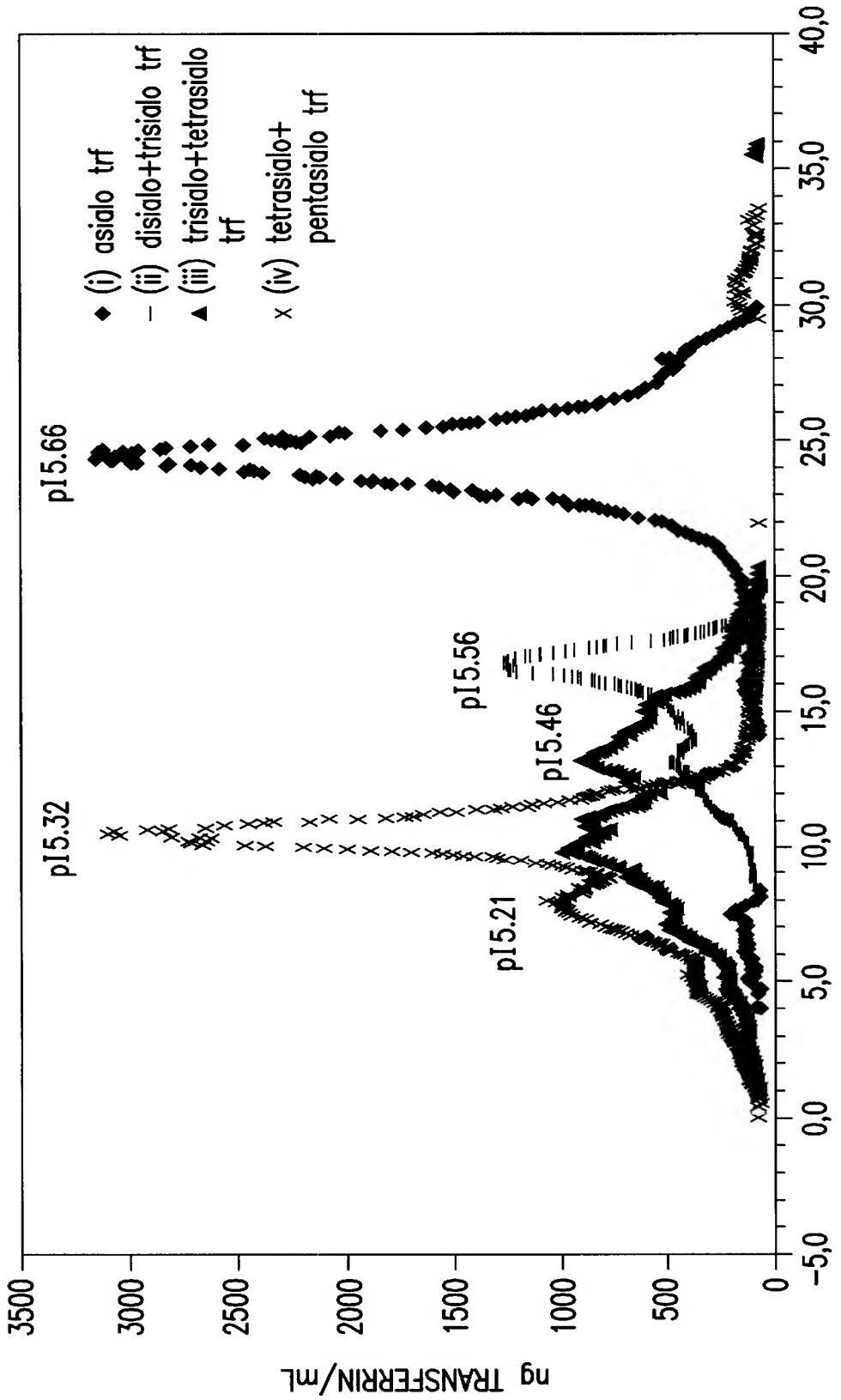
FIG.3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(iii) trisialo+tetrasialo trf
(iv) tetrasialo+ pentasialo trf

FIG.4

ANALYTICAL METHOD AND APPARATUS

This application claims the benefit of No. 60/148,566, filed Aug. 13, 1999.

The present invention relates to a method and apparatus for determining analytes in a sample, and more particularly to a method and apparatus where the analytes are separated prior to detection.

BACKGROUND OF THE INVENTION

Biomolecules may be present in several heteroforms, such as isoforms, where small changes in the molecular structure may cause great changes in the effect of the molecule. Such small structural changes may, however, be difficult to measure specifically, even with methods of high specificity, such as immunoassays, as the compounds usually will compete for the binding to a specific antibody. In our copending international (PCT) application WO 99/60402, such structural changes are discussed and a method is disclosed for measuring some of the heteroforms, for example those having the highest positive or negative charge. The method in question uses a flow matrix having an application zone for sample and, downstream thereof, a detection zone with immobilized reagent which binds analyte and where bound analyte is detected. A separation zone is provided between the sample application zone and the detection zone. In the separation zone, disturbing components or components not to be determined are bound or retarded and prevented from reaching the detection zone with the analyte. If, for example, the analyte is one of two heteroforms, the other heteroform, which is not to be determined but would compete with the analyte for binding in the binding zone, is retarded in the separation zone to permit selective detection of the analyte. There may, however, often be more than two heteroforms. For example, transferrin may exist in at least nine different isoforms, where a few of the isoforms, primarily disialo transferrin but also asialo transferrin, are important to measure for testing alcohol abuse. To be able to measure all the isoforms in a complex mixture, it has so far been necessary to separate the isoforms by column chromatography, and then analyze each fraction for the presence of an isoform by spectrophotometric or immunoassay detection depending on the concentrations of the analytes to be measured.

WO 99/30145 discloses 2-dimensional gel electrophoresis for qualitative determination of nucleic acids, proteins, carbohydrates or lipids in a sample. The gel contains a separation gel with a sample loading zone and provided in a slot within the separation gel, a detection gel having an immobilized probe for one or more target molecules. After electrophoretic separation in the separation gel in a first dimension, the gel is rotated 90 degrees and electrophoresis is performed in a second dimension to transport the target molecule to the detection zone where binding of the target molecule to the immobilized probe is detected. There is no suggestion in WO 99/30145 that heteroforms could be determined. Also, electrophoretic systems are generally rather laborious and often expensive, especially when an additional detection step is to be included in the electrophoretic system.

U.S. Pat. No. 4,469,601 discloses a method and system for multi-dimensional chromatography in a thin-layer chromatographic plate wherein a sample is separated into an array of constituents. These constituents are then separated into a second array of sub-constituents by pumping a fluid through the plate in a direction crossing the array, and the sub-constituents are detected as they flow past fixed positions in this second direction. Thin layer chromatography is, however, restricted to the separation of small (i.e. low molecular weight) molecules, and does not permit the separation of biomolecules, such as proteins, for example.

Pristoupil, T. I., Chromatog. Rev., 12 (1970) 109–125 describes the use of nitrocellulose filters in chromatography and electrophoresis. Chromatography in aqueous solution was performed with a nitrocellulose membrane in a horizontal position in a plexiglass chamber. Proteins were detected by immersing the membrane in a staining solution, and other substances were detected by usual spray or sandwich techniques. On the intact membrane, proteins having a molecular weight of the order of 10^5 and higher were firmly adsorbed on the membrane while peptides, amino acids and other low-molecular substances of hydrophilic character migrated with the front of the developing solution. For electrophoresis, it was necessary to impregnate the membrane with neutral detergents to prevent the high adsorption of proteins. Also immunochromatography of rabbit anti-bovine serum and immunochemically inactive normal rabbit serum on a membrane with bovine serum adsorbed thereto is described. The antigen-antibody complex gave a distinct spot at the start, while the immunochemically inactive proteins migrated without any marked adsorption. Thus, no "true" chromatography of components seems to have been obtained neither in the intact (or plain) membrane nor in the antibody-coated membrane but rather either firm binding or no binding at all.

There is therefore a need for an analytical method and apparatus which permit the determination of heteroforms of biomolecules and by which assays may be performed more quickly and more easily than by the prior art methods and apparatuses, respectively.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a determination method which overcomes the shortcomings of the prior art methods and which readily permits quick and reliable determination of all heteroforms of biomolecules, such as isoforms, even in a complex sample. It is a further object of the present invention to provide a determination method which may be performed on a single plate, sheet or chip. Still another object of the present invention is to provide a simple and easy-to-use apparatus of plate, sheet or chip type for performing the method of the present invention.

According to the present invention, the above and other objects and advantages are obtained by a method wherein analytes (such as isoforms) in an aqueous sample are separated in a flow matrix which permits capillary force assisted fluid flow therethrough, especially a planar flow matrix such as a chromatographic membrane (e.g. an ion exchange membrane). To determine the separated analytes, this being the gist of the invention, the separated analytes are eluted from the separation area of the flow matrix in a direction substantially transverse to the separation direction to migrate to a capture zone with immobilized reactant (such as a single immobilized antibody common to all the analytes), usually in the form of a line or band, where the eluted analytes are captured. There, the analytes may be detected and determined by the addition of a detection reagent capable of binding to the captured analytes. The detection reagent may e.g. be a suitably labelled antibody directed to the isoform, such as an antibody labelled by a black-coloured particle. In the latter case, for example, the varying colour intensity along the detection line or band may be readily detected and quantified by means of a scanner.

Essentially aqueous systems are used in the separation and elution steps. "Essentially aqueous" means here that the system is either completely aqueous or may contain a small amount, not more than about 3%, of one or more other solvents. Preferably, about 99%, more preferably about 99.5%, usually at least about 99.9% of the essentially aqueous system is water.

Thus, in one aspect the present invention provides a method for qualititative, semi-quantitative or quantitative determination of at least two analytes in an aqueous sample containing or suspected of containing said analytes, said method comprising the steps of:

(i) providing a flow matrix comprising a separation zone extending in a first dimension thereof, and a detection zone extending in said first dimension in a spaced parallel relationship with the separation zone, said detection zone comprising an immobilized reagent capable of capturing said analytes through biospecific interaction therewith,

(ii) applying said sample to the flow matrix at or upstream of said separation zone,

(iii) initiating a first essentially aqueous fluid flow in the flow matrix along the separation zone in said first dimension to transport said analytes through said separation zone to be separated therein,

(iv) interrupting said first fluid flow and initiating a second essentially aqueous fluid flow in a second dimension of the flow matrix substantially transverse to said first dimension towards the detection zone, to transport said separated analytes to the detection zone to be captured therein by said immobilized reagent, and

(v) determining said analytes in said detection zone.

In another aspect, the present invention provides an apparatus for carrying out the method of the invention, which apparatus comprises:

(i) a flow matrix having a separation zone and a detection zone extending in a spaced parallel relationship in a first dimension of the flow matrix, wherein the detection zone comprises immobilized reagent capable of binding the analytes through specific interaction therewith,

(ii) means for initiating a first essentially aqueous fluid flow in the flow matrix along the separation zone in said first dimension of the flow matrix,

(iii) means for initiating a second essentially aqueous fluid flow in a second dimension of said flow matrix substantially transverse to the said first dimension towards the detection zone,

such that when a sample containing the analytes is introduced into to the separation zone, the analytes may be separated in the separation zone by said first fluid flow through the separation zone and transported by said second fluid flow to the detection zone where the analytes are bound to the immobilized reagent to be determined.

Preferably, the flow matrix is at least substantially planar.

The separation zone and the detection zone (which may be integral or two separate parts joined to each other) may either be arranged in the same plane of the flow matrix, or be arranged on top of each other. In the latter case, the two zones must be prevented from contacting each other such as by a removable partitioning element when the separation phase of the method of the invention is performed. Such a separating element may be a film or the like that is removed prior to performing steps (iv) and (v) above.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic planar view of an embodiment of apparatus according to the present invention.

FIGS. **2**A, **2**B and **2**C are schematic planar views of the apparatus in FIG. **1**, each illustrating a different stage in the method of the present invention.

FIG. **3** is a diagram showing the detected intensity curves for transferrin isoforms analyzed by the method of the present invention.

FIG. **4** is a diagram showing four superposed intensity curves for detected transferrin isoforms from separate analyses by the method of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, the method of present invention is particularly useful for determining heteroforms of biomolecules, i.e. closely related biomolecular analytes, which may not be distinguished by a specific ligand or receptor, such as an antibody. Exemplary heteroforms include isoforms of proteins, e.g. differently glycosylated proteins (glycoproteins) where small variations in the carbohydrate structure can give isoforms with different isoelectric points, isoenzymes, etc. The term heteroforms also includes inter alia different forms of bioaffine complexes, where one part of the complex belongs to the isoform protein, e.g. free and antibody bound molecules. A so-called inhibition test may be used to determine if two compounds are heteroforms to one another. Reacting the ligand with one or both of the suspected isoforms and comparing the result, makes it possible to decide if the molecules are isoforms.

Glycoproteins such as transferrin, FSH, LH and TSH are examples of analytes that occur in a variety of isoforms, the relative occurrence of which is of clinical importance but which usually are not possible to differentiate by immunoassays as they are very similar from an immunochemical point of view. Other examples of present interest are so-called cardiac markers (e.g. creatine kinases) which occur in different isoforms with different charges as a result of protolytic degradation in the extracellular milieu.

Transferrin and their isoforms of interest are present in rather high concentrations in blood and can be analysed by spectrophotometric on-line detection (Jeppsson, J.-O., Clin. Chem. 39/10, 2115–2120 (1993)) directly after column separation, if enough amount of sample is applied on the column. Other molecules such as the hormones FSH, LH and TSH are present in low concentrations which require immunoasssay detection (Wide, L., Acta Endocronologica 1985, 109: 181–189).

In the method of the invention, the separation of analytes such as isoforms may be performed by applying the sample containing the isoforms on a planar flow matrix, especially a membrane, separating the isoforms by a first liquid flow in a separation area of the flow matrix, and then eluting the separated isoforms through a second liquid flow transverse to the first liquid flow such that the the isoforms are removed from the separation area and pass a detection area containing immobilized capturing reagent, usually as a line or band, to be captured thereby. The captured isoforms may then be detected by the application of a detecting reagent, e.g. a labelled antibody to the isoform which, for example, may be added via a third fluid flow in the same, opposite or transverse direction to that of the above-mentioned second flow.

In a (presently less preferred) embodiment, the separation zone and the detection zone may be provided on two separate flow matrix members placed on top of each other and separated by a removable film or the like which is removed prior to elution of the separated analytes and transport thereof to the detection zone.

A planar flow matrix, such as a membrane, designed for such an analysis comprises a separation zone, and in parallel and spaced thereto, a detection zone containing one or more lines or bands of immobilized capturing reagent extending along the detection zone.

As is readily seen from the above, and as will be better understood from the following description, the present invention offers a method and apparatus for rapidly analysing isoforms of proteins and other heteroforms, which has not been possible to achieve before.

Flow Matrix

The material of the flow matrix (including the separation zone and the detection zone) may be of the same type as that previously utilized in so-called immunochromatographic determination methods and defines the room in which sample components (including analytes) and reactants are transported, provided, of course, that the material permits flows in different directions. The inner surface of the matrix, i.e. the surface of the flow channels in the matrix, should be sufficiently hydrophilic to permit aqueous media, such as buffer, serum, plasma, blood, saliva etc, to be transported through the matrix. This transport may be achieved or assisted by capillary forces, either by capillary forces in the matrix itself or in an auxiliary means, such as an absorbent element (e.g. a sucking pad of cellulose or the like) brought in contact with matrix. The capillary flow may optionally be further assisted by pressure or suction applied by a pump device. The smallest inner dimension (for round channels measured as a diameter) should be sufficiently great to permit transport through the matrix of analyte and added reactants, Typically, suitable matrices may be selected among those having flow channels of a smallest inner dimension in the range of 0.01–1000 μm, with preference for 0.4–100 μm if the matrix has a system of communicating flow channels. Flow channels having their smallest dimension in the upper part of the broad range (up to 1000 μm) are primarily useful for flows driven by externally applied pressure/suction.

Presently, it is preferred that the flow matrix is in the form of a membrane, usually with a thickness less than about 500 μm, e.g. in the range of from about 25 μm to about 500 μm, and preferably less than about 150 μm, e.g. in the range of from about 75 to about 150 μm. Other types of matrices may, however, also be contemplated, such as a gel or a silicon (or glass) plate or chip with etched interconnected channels, etc. as is per se well known in the art.

Suitable matrices are often built up from a polymer, for example nitrocellulose, polyester, polyethersulphone, nylon, cellulose nitrate/acetate, cellulose, regenerated cellulose. Advantageously, membranes of such materials may be provided with a tight backside or backing of e.g. polyester.

The homogeneity of the flow matrix material affects its chromatographic quality and may therefore be reflected in terms of theoretical plate height. The lower height of the theoretical plate, the better the material. For example, a membrane for use in the present invention should preferably have a height of theoretical plate (HETP) of less than about 500 μm, especially less than about 100 μm.

Separation Zone

The separation zone and the detection zone may be integral parts of one and the same flow matrix or may be an assembly of separate parts. The separation zone, which optionally may comprise two or more subzones, may be based on various principles, permitting essentially aqueous systems to be used, including ion-exchange chromatography, chromatofocusing, gel filtration (size separation) (e.g. using a gel or a dense membrane), affinity (preferably a moderate binding constant of $<10^6$, especially 10^3–10^6), including e.g. IMAC (immobilized metal chelate affinity chromatography), and hydrophobic interaction chromatography (HIC).

The separation zone exhibits a ligand/structure having a certain binding capability for the desired sample components (analytes and related heteroforms). The choice of ligand or structure, especially with regard to specificity, binding strength (affinity), and kinetics to suit the purposes of the present invention are readily apparent to a person skilled in the art. Ligands that make separation in the separation zone possible may thus be charged (anionic, cationic, amphoteric=ion-exchange ligands), amphoteric/amphiphilic, bioaffine, chelating, sulphur-containing (primarily thioether for so-called thiophilic affinity), or based on π-π interaction, hydrophobic etc. Among biospecific affinity ligands, primarily so-called immunoligands are noted, i.e. antibodies and antigen-binding fragments thereof.

The ligands/structures in question may be structures physically introduced into the matrix in the manufacturing process, or may be anchored to the separation zone, either by covalent binding to the matrix, or via physical adsorption. The anchorage of the ligands/structures to the matrix may take place via a polymer or other substituent which in turn carries covalently, physically adsorptively, or biospecifically bound ligands that are used in the separation. Another possibility is deposition of polymer particles which exhibit a desired type of ligand. The particles may be of hydrophilic or hydrophobic character, and the ligand structure may be exhibited by a compound adsorbed or covalently bound to the particles. Regarding the technique for binding a separating ligand to the matrix, it may, for example, be referred to our previously filed International (PCT) applications WO 99/36780, WO 99/36776 and WO 99/36777 (the disclosures of which are incorporated by reference herein). In this connection it may be mentioned that there are commercially available membranes which have covalently bound ligands, for example DEAE cellulose paper (diethylaminoethyl) (DE81, Whatman International Ltd, England).

The ligand density (substitution degree) is selected to obtain the desired isocratic separation. Optionally, the separation zone may have different ligand densities or a gradient of ligand densities along the separation direction.

Examples of ion-exchange functional groups include anion exchangers, such as diethyl aminoethyl (DEAE), trimethyl hydroxypropyl (QA), quaternary aminoethyl (QAE), quaternary aminomethyl (Q), diethyl-(2-hydroxypropyl)-aminoethyl, triethyl aminomethyl (TEAE), triethylaminopropyl (TEAP), polyethyleneimine (PEI), and cation-exchangers, such as methacrylate, carboxymethyl (CM), orthophosphate (P), sulfonate (S), sulfoethyl (SE), sulfopropyl (SP).

After the ligand coating, the membrane is usually treated with a detergent or other suitable agent to substantially reduce or eliminate undesired background or unspecific adsorption effects of the membrane matrix as is per se known in the art.

The sample containing the analytes may be added directly on the flow matrix surface, but usually it is added to a separate sample application membrane or pad in liquid contact with the membrane, either in edge to edge contact therewith or, preferably, mounted on top of the flow matrix.

The conditions for the separation of the analytes in the separation zone is selected depending on the separation principle used, but generally the conditions are isocratic or with stepwise or continuously changed ion-strength. On the other hand, the transverse elution of the analytes from the

separation zone is usually performed at isocratic conditions. Thus, in for example gel filtration, the separation buffer and elution buffer may be the same, whereas in ion-exchange chromatography it is normally necessary to use an elution buffer of high ionic strength for efficient elution of the separated analytes.

Detection Zone

In the detection zone, the analyte capture may be based on different principles such as biospecific capture, preferably immunochemical, or group-specific capture, e.g. binding of proteins based on the presence of hydrophobic groups. Presently, biospecific capture is preferred, a binding constant of $K>10^8$ then being desirable.

The capture reagent be bound to or immobilized in the detection part of the flow matrix as is well known in the art, e.g. in the same way as described above for the ligands in the separation zone.

As mentioned above, the detection zone preferably comprises a continuous line or band of capture reagent. In the case of a high concentration of one or more of the analytes, it may be necessary to use two, or occasionally even more detection lines or bands. Analogously, the ligand density in the detection zone may be varied depending on the mutual concentrations of the different analytes.

The detection zone should, of course, be sufficiently spaced from the separation zone for analyte not to spread to the detection zone before the separation is complete.

While it is desired that the release and transport of analyte from the separation zone to the detection zone be substantially complete, this is not necessary for the binding in the detection zone, provided that all the analytes bind to the same degree in the detection zone.

Depending on the type of flow matrix used, the elution flow from the separation zone may be guided to the detection zone through suitable delimiters, such as wax delimiters, laser made grooves etc.

The separation zone and the detection zone may be separate parts of different materials joined to form a combined flow matrix, but the two zones may, however, also be provided on an integral matrix, such as membrane or chip, by suitable chemical/physical modification thereof as is per se known in the art.

Detection Methods and Reagents

Detection and quantification of the analytes captured in the detection zone may take place in various ways. If the captured analytes are enzymatically active, they may be detected by their action on a suitable substrate, e.g. a colour change. Usually, however, a detectable reagent is added. Such a substrate or reagents may be added via a fluid flow in the matrix, either (i) from one of the sides transverse to the separation direction of the flow matrix (usually a long side), preferably in the opposite direction of the elution flow, or (ii) from one of the sides extending in the separation direction of the flow matrix (usually a short side), or (iii) on top of the matrix, usually near the detection zone via a pad or foldable part of the matrix such that the substrate or reagents may be transported by a fluid flow into the detection zone. In the last-mentioned case, a diffusive detection reagent may optionally be pre-deposited in the pad or foldable part. Such pre-deposition and folding structures are per se well known in the art. Excess of substrate or reagents will be removed by a buffer flow.

The detectable reagent is usually a biospecific affinity reactant which is labelled with an analytically detectable group, such as an enzymatically active group (e.g. colour formation upon action on substrate), fluorescent group, chromogenic group, hapten, biotin, radiolabel (autoradiography), particles, etc. A usual form of analytically labelled reactants is labelled antibody.

Labelled antibody may be used in (i) non-competitive techniques, such as sandwich technique, in which the capturer is an antibody which may be directed against the same antigen (=analyte) as the labelled antibody, or an antigen/hapten, or (ii) competitive techniques in which competition takes place between analyte and solid phase-bound analyte analogue for a limiting amount of anti-analyte antibody.

A particularly useful labelling group is particles, for example black-coloured carbon particles which may be measured directly, e.g. with a conventional type scanner. Optionally, the particles contain one of the above mentioned detectable groups, such as fluorophoric group or chromogenic group (fluorescent and coloured particles, respectively). Useful particles often have a size in the range 0.001 to 5 μm, with preference for the range 0.05 to 5 μm. The particles may be of colloidal dimensions, so-called sol (i.e. usually spherical and monodisperse having a size in the range 0.001 to 1 μm). Especially may be mentioned metal particles (for example, gold sol), non-metal particles (for example SiO_2, carbon, latex and killed erythrocytes and bacteria). Also particles of non-colloidal dimensions have been used. These have been more or less irregular and more or less polydisperse (for example, carbon particles <1 μm; see e.g. our WO 96/22532).

When particles are the label group, the complexes formed in the detection zone may often be detected visually or by optical measuring equipment (e.g. a CCD camera coupled to a computer with special software for image analysis or laser scanner).

For particles as label group, it may further be referred to e.g. WO 88/08534 (Unilever); U.S. Pat. No. 5,120,643 (Abbott Labs.); EP-A-284,232 (Becton Dickinson).

The invention is primarily intended for biological samples, for example, blood (serum, plasma, whole blood), saliva, tear fluid, urine, cerebrospinal fluid, sweat, etc. The invention is also applicable to other samples, such as fermentation solutions, reaction mixtures, etc.

ILLUSTRATIVE EMBODIMENT

In order to facilitate the understanding of the present invention, an embodiment thereof will now be described in more detail, by way of example only, with reference to FIGS. **1** and **2**A to **2**C of the drawings.

FIG. **1** illustrates schematically a membrane that may be used for the analysis of e.g. isoforms of proteins or other heteroforms in accordance with the method of the invention. The membrane consists in the illustrated case of two combined parts of different materials, a separation part **1** and a detection part **2**, joined by a piece of adhesive tape (not shown) on the backside of the combined membrane and in liquid receiving contact with each other by a.thin membrane band **3** as an overlap. This membrane band **3** is secured to the separation/detection membrane by a piece of adhesive tape **4**. The separation part defines a separation zone on the combined membrane. The detection part **2** has a detection zone in the form of a line **5** of immobilized capture reagent for analytes, e.g. having a width of about 1 mm. Reference numeral **6** indicates an optional additional detection line to increase the measurement range. The short-sides of the membrane are indicated in FIG. **1** by a and c and the long-sides by b and d, respectively.

The membrane may be used as follows with reference to FIGS. **2**A to **2**C. After wetting the membrane, a sample containing two analytes to be analysed (referred to as analytes **1** and **2** below) is applied at **7** on the separation zone

1 (FIG. 2A). A pad **8** containing separation buffer is applied at short-side a of the membrane and a sucking pad **9** at the opposite short-side c. This will cause a buffer flow in the direction of the arrow in FIG. **2**A, separating the two analytes as indicted by the dots at **10** (analyte **1**) and **11** (analyte **2**) in FIG. **2**A.

With reference to FIG. **2**B, pads **8** and **9** are then removed and an eluent-containing pad **12** is mounted to the long-side d, and a sucking pad **13** is mounted to long-side b. This causes a flow of eluent in the direction of the arrow in FIG. **2**B, transporting the separated analytes **1** and **2** to the detection zone where they are captured by the immobilized reagent line at positions **14** and **15**, respectively, along the line.

Then, with reference to FIG. **2**C, the pads **12** and **13** are removed and replaced by a sucking pad **16** at the long-side d, and a container **17** with a solution or suspension of labelled reactant at the long-side b. Thereby, labelled reactant will migrate in the direction of the arrow and bind to the captured analytes **1** and **2** at **18** and **19** in FIG. **2**C. The labelled complexes, and thereby the corresponding analytes **1** and **2**, may then be detected and quantified by reading the intensity of the signals from the label along the detection line and calculating the respective amounts. In case the label is carbon particles, the measurements may advantageously be performed with a scanner.

The above described manual initiation and stopping of the flows are, of course, only given for purpose of illustration, and more sophisticated means therefor are readily apparent to a person skilled in the art, such as, for example, so-called imprinted liquid circuits (see e.g. WO 93/10457) etc.

A specific example where the method of the present invention is used for the analysis of isoforms of transferrins is described below.

EXAMPLE 1

Determination of Isoforms of Transferrin

(i) Preparation of Separation Membrane with Anion-exchanging Properties

A sheet of nitrocellulose membrane (3 μm, nitrocellulose on polyester backing, Whatman International Ltd, England) was placed in a solution of 0.03% polyethyleneimine (PEI, Sigma, St Louise, Mo., USA). The mixture was shaken for three hours and the membrane was then placed in 0.1% Tween 20 for 30 minutes, dried in air and then stored in a plastic bag at +4° C.

(ii) Preparation of Detection Membrane

Anti-transferrin monoclonal antibody, 3 mg/ml, was sprayed onto strips (29 cm×4 cm) of nitrocellulose membranes (5 μm, on polyester backing, Whatman International Ltd, England) in two 1 mm broad lines in the centre of the strip, separated by 2 mm and in parallel with the long side of the strip. The spraying equipment (IVEK Linear Striper IVEK Corporation, Vermont, USA) delivered 1 μl/cm. The membranes were dried at room temperature and stored in a plastic bag at +4° C.

(iii) Preparation of Combination Membrane

The separation membrane was cut to 1.5×5 cm and the detection membrane was cut to 2×5 cm such that the two antibody lines were located centrally on the membrane and in parallel with the long side. The two membranes were put tightly together along the long sides and joined by means of adhesive tape on the underside. A piece of nitrocellulose membrane (0.2 cm×5 cm, 8 μm, A99, Schleicher and Shuell, Dassel, Germany) was placed on the top side of the two membranes as an overlap. This membrane was anchored by means of a 1×4 cm self-adhesive polyester film (Gelman adhesive polyester film, 3 mil) placed such that 0.5 cm at the short side end on the formed combined separation/detection membrane remained uncovered. Below, the two short sides of the combination membrane are referred to as a and c, respectively, and the two long sides as b and d, respectively (see FIG. **1**).

(iv) Carbon Particle Conjugate

Carbon Particle Suspension (Stock Solution)

3 g of carbon particles (sp 4, Degussa, Germany) were suspended in 250 ml of 5 mM borate buffer, pH 8.4, and sonicated (VibraCell 600 W, 1.5 cm probe) in an ice-bath for 4×5 minutes at 100% amplitude and 5+5 seconds pulse.

Carbon Particle Conjugate

75 μg/ml of anti-transferrin monoclonal antibody and carbon suspension (250 μg/ml) were mixed for 3 hours. Bovine serum albumin (BSA), corresponding to 1% final concentration, were added and the particles were mixed for additionally 30 minutes and then washed by means of centrifugation and decanting in 0.1 M borate buffer, pH 8.5 (containing 1% BSA and 0.05% NaN_3) and diluted to 3.7 mg carbon/ml with wash buffer. The ready carbon particle conjugate was stored at +4° C. in wash buffer.

(v) Sample Materials

Tetrasialo Transferrin, Trisialo Transferrin and Disialo Transferrin

These iso-transferrins were isolated from an iron-saturated preparation of transferrin (Sigma, St Louis, Mo., USA) by ion-exchange chromatography on Mono Q (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Asialo Transferrin

An iron-saturated preparation of transferrin (Sigma, St Louis, Mo., USA) was treated with neuraminidase (Behring ORKD, Germany), and asialo transferrin was then isolated by ion-exchange chromatography on Mono Q (Amersham Pharmacia Biotech AB, Sweden).

The various isoforms were diluted in 0.2% BSA, 0.1% bovine gammaglobulin, 0.1% Tween 20, 0.1 mM Fe^3+-citrate, 1 mM $NaHCO_3$ and 0.05% NaN_3 to the concentrations 2–6.5 μg transferrin/ml.

Isoelectric Points (pI)

The pI's were determined for the respective isoform preparation by repeated isoelectric focusing in Phast System (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Asialo transferrin pI=5.66; disialo transferrin pI=5.56; trisialo transferrin pI=5.46; tetrasialo transferrin pI=5.32 and pentasialo transferrin pI=5.21. Amersham Pharmacia Calibration Kit, 17-0472-01, 2.5–6.5 was used for calibration.

Transferrin Standard

Asialo transferrin prepared as described above was diluted in 20 mM Bis-Tris, pH 6.37, containing 0.2% BSA, 0.1% Tween 20, 0.1 mM Fe^3+ citrate, 1 mM $NaHCO_3$ and 0.05% NaN_3 to the concentrations 0.07–16.6 μg transferrin/ml and was used as a standard.

(vi) Standard Protocol for Combined Separation and Immunochemical Determination

Step 1. Wetting of Membrane from Short Side a to Short Side c

The combination membrane is wetted by adding elution buffer to a 1×3.5×0.5 cm PVA sponge (PVA D, 60 μm,

Kanebo Ltd, Japan) and then placing the sponge along short side a of the pad. To the opposed short side c of the membrane is mounted a 2×3.5 cm sucking cellulose pad (GB 004, Schlecher and Schuell). When the elution buffer front has reached the cellulose pad, the PVA sponge is removed. For analysis (a) below, the elution buffer was 20 mM Bis-Tris, 0.1% Tween 20, 5 mM NaCl, pH 6.12; and for analysis (b) below, the elution buffer was 20 mM Bis-Tris, 0.1% Tween 20, 15 mM NaCl, pH 6.32.

Step 2. Sample Application and Elution from Short Side a to Short Side c

0.5 μl of sample (2–6.5 μg/ml) is placed on the middle of the separation membrane, 0.5 cm from the short side a. The PVA sponge with elution buffer is added and the elution is continued for 4 minutes. Then the PVA sponge and the sucking pad are removed.

Step 3. Elution from Long side d (Separation Membrane) to Long Side b (Detection Membrane)

Along long side b (detection membrane) is mounted a 2×5 cm cellulose pad (GB 004, Schlecher and Schuell), and along long side d is placed a 1×5×0.5 cm PVA sponge (PVA D, 60 μm, Kanebo Ltd, Japan) wetted by elution buffer (20 mM Bis-Tris, 200 mM NaCl, 0.1% Tween 20, pH 6.29). The elution is continued for 4 minutes and the flow is stopped by removing the PVA sponge and the sucking pad.

Step 4. Reaction with Carbon-anti-transferrin

A 2×5 cm sucking cellulose pad (GB 004, Schlecher and Schuell) is mounted along long side d (separation membrane part), and then long side b is placed in a container with carbon-anti-transferrin, 0.25 mg carbon/ml in 40% trehalose, 1% Tween 20, 1% bovine albumin, 0.1 M borate buffer, pH 8.5, 0.05% NaN_3. The carbon particle conjugate is allowed to pass the detection lines for 2 minutes, and the combination membrane is then removed from the container, the sucking pad is removed and the combination membrane is left to dry.

Step 5. Detection of Blackening and Calculation of Transferrin Concentration

The membrane is placed in a scanner (Agfa Acus II Scanner) for mesurement of a grey scale along the detection lines. The grey scale is read with a 12 bits grey scale resolution (4096 levels) and 600 points per inch (ppi) optical resolution. The image obtained is digitalised and the intensity values are processed by means of Microsoft Excel. The average value of the intensity along the short side of the detection line (1 mm=23 grey scale values) are calculated and the chromatogram for 4 cm along the detection line may be illustrated graphically.

For calculating the concentration of transferrin, a dilution series of asialo transferrin is used where 0.5 μl of sample has been dispensed onto the separation membrane and then directly eluted off (steps 3–5). The top intensity of the respective standard point is measured, a standard curve is constructed by means of a curve-fitting program (GraphPad Prism® nonlinear fit) and the transferrin concentration for the chromatograms may be calculated.

(vi) Analyses

a) A prepared sample containing asialo transferrin, disialo transferrin, trisialo transferrin and tetrasialo transferrin was analysed according to the standard protocol above, and the signal intensity curves obtained are shown in FIG. **3**. In the diagram, numeral **1** indicates the peak for tetrasialo transferrin, **2** is trisialo transferrin, **3** is diasialo transferrin, and **4** is asialo transferrin.

b) prepared samples containing (i) asialo transferrin, (ii) disialo+trisialo transferrin, (iii) trisialo+and tetrasialo transferrin, and (iv) tetrasialo+pentasialo transferrin, respectively, were analysed according to the standard protocol above, and the results are shown in FIG. **4**. The different peaks are identified in the top right hand corner of the diagram. Also the isoelectric point values of the peaks are indicated.

As demonstrated by FIGS. **3** and **4**, the method of the invention permits excellent separation and quantification of isoforms in a sample. For example, a resolution of 0.1 pI unit is readily achieved as shown in FIG. **4**.

While the invention has been described and pointed out with reference to operative embodiments thereof, it will be understood by those skilled in the art that various changes, modifications, substitutions and omissions can be made without departing from the spirit of the invention. It is intended therefore that the invention embraces those equivalents within the scope of the claims which follow.

What is claimed is:

1. A method for qualitative, semi-quantitative or quantitative determination of at least two analytes in an aqueous sample containing or suspected of containing the analytes, said method comprising the steps of:

(i) providing a flow matrix comprising a separation zone extending in a first dimension thereof, and a detection zone extending in said first dimension adjacent the separation zone, said detection zone comprising an immobilized reagent capable of capturing said analytes through biospecific interaction therewith,

(ii) applying said sample to the flow matrix at or upstream of the separation zone,

(iii) initiating a first essentially aqueous fluid flow in the flow matrix along the separation zone in said first dimension to transport the analytes through the separation zone to be separated therein,

(iv) interrupting said first fluid flow and initiating a second essentially aqueous fluid flow in a second dimension of the flow matrix substantially transverse to said first dimension towards the detection zone to transport said separated analytes to the detection zone to be captured therein by said immobilized reagent, and

(v) determining the analytes in the detection zone,

wherein the fluid flows in said matrix are assisted by capillary forces, and

wherein the fluid transport in the flow matrix is at least assisted by an absorbent element which may be separate from or integral with the flow matrix.

2. The method according to claim **1**, wherein the flow matrix is at least substantially planar.

3. The method according to claim **1** or **2**, wherein the separation zone and the detection zone are arranged in the same plane and said first and second fluid flows are lateral flows.

4. The method according to claim **1** or **2**, wherein the separation zone and the detection zone are arranged on top of each other and said first fluid flow is lateral and said second fluid flow is in depth in the flow matrix.

5. The method according to claim **1**, wherein the fluid transport in the flow matrix at least partially is effected by capillary forces in the matrix itself.

6. The method according to claim **1**, wherein the flow matrix is wetted by an essentially aqueous fluid prior to starting the assay.

7. The method according to claim **1**, wherein the determination of analytes (v) comprises reacting said captured analytes with a detectable reagent.

8. The method according to claim **7**, wherein the detectable reagent is an immunochemical reagent.

9. The method according to claim 1, wherein the determination of analytes (v) comprises reacting unreacted immobilized reagent in the matrix with detectable analyte or a detectable analyte analogue.

10. The method according to claim 7, 8 or 9, wherein said detectable reagent, analyte or analyte analogue comprise a detectable label.

11. The method according to any one of claims 7 to 9, wherein said detectable reagent, analyte or analyte analogue is introduced by a third fluid flow.

12. The method according to claim 11, wherein said third fluid flow is a lateral flow applied from one side of said flow matrix.

13. The method according to claim 1, wherein the separation in said separation zone is based on chromatography.

14. The method according to claim 13, wherein said chromatography is selected from the group consisting of ion-exchange chromatography, chromatofocusing, size exclusion chromatography, affinity chromatography, and hydrophobic interaction chromatography.

15. The method according to claim 1, wherein said separation zone comprises a gradient in said first direction with regard to separating capability.

16. The method according to claim 1, wherein said analytes are heteroforms of a biomolecule.

17. The method according to claim 16, wherein said heteroforms have different charges.

18. The method according to claim 16 or 17, wherein said analytes are glycoproteins.

19. The method according to claim 1, wherein said flow matrix comprises a membrane.

20. The method according to claim 1, wherein said matrix comprises a membrane having particles deposited therein.

21. The method according to claim 1, wherein said detection zone comprises at least two parallel detection lines or bands containing immobilized reagent.

22. The method according to claim 1, wherein the binding of the analyte in the detection zone is by an immunochemical interaction.

23. An apparatus for determining analytes in a sample, which apparatus comprises:

(i) a flow matrix having a separation zone and a detection zone extending in a spaced parallel relationship in a first dimension of the flow matrix, wherein the detection zone comprises immobilized reagent capable of binding the analytes through biospecific interaction therewith,

(ii) means for initiating a first essentially aqueous fluid flow in the flow matrix along the separation zone in said first dimension of the flow matrix,

(iii) means for initiating a second essentially aqueous fluid flow in a second dimension of said flow matrix substantially transverse to the said first dimension towards the detection zone,

such that when a sample containing the analytes is introduced into to the separation zone, the analytes may be separated in the separation zone by said first fluid flow through the separation zone and transported by said second fluid flow to the detection zone where the analytes are bound to the immobilized reagent to be determined.

24. The apparatus according to claim 23, wherein the flow matrix permits capillary force assisted fluid flow therethrough.

25. The apparatus according to claim 23 or 24, wherein the flow matrix is at least substantially planar.

26. The apparatus according to claim 23 or 24, wherein the separation zone and the detection zone are arranged in the same plane and said first and second fluid flows are lateral flows.

27. The apparatus according to claim 23 or 24, wherein the separation zone and the detection zone are arranged on top of each other and said first fluid flow is lateral and said second fluid flow is in depth in the flow matrix.

28. The apparatus according to claim 23, wherein the separation zone is based on choromatography selected from the group consisting of ion-exchange chromatography, chromatofocusing, size exclusion chromatography, affinity chromatography, and hydrophobic interaction chromatography or comprises a gradient in said first direction with regard to separating capability.

29. The apparatus according to claim 23, wherein the flow matrix comprises a membrane or a membrane having particles deposited therein.

30. The apparatus according to claim 23, wherein the detection zone comprises at least two parallel detection lines or bands containing immobilized reagent.

31. The apparatus according to claim 23, which apparatus comprises means for creating liquid suction in the flow matrix.

32. The apparatus according claim 31, wherein said means for creating liquid suction comprise an absorbent member at the downstream end of the flow matrix.

33. The apparatus according to claim 23, which apparatus comprises means for supplying flow liquid to the flow matrix.

* * * * *

US005561097A

United States Patent [19]

Gleason et al.

[11] **Patent Number: 5,561,097**

[45] **Date of Patent: Oct. 1, 1996**

[54] **METHOD OF CONTROLLING DENSITY OF LIGAND COUPLED ONTO SUPPORTS AND PRODUCTS PRODUCED THEREFROM**

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[21] Appl. No.: **234,654**

[22] Filed: **Apr. 28, 1994**

[51] **Int. Cl.**[6] **B01J 20/26**

[52] **U.S. Cl.** **502/402**; 502/401; 502/403; 502/159; 502/162; 502/168; 502/172

[58] **Field of Search** 502/401, 402, 502/403, 159, 162, 168, 172

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[57] **ABSTRACT**

A method is disclosed for controlling the coupling low molecular weight ligands to activated sites on a support in competition with a quencher of the activated sites. Derivatized supports produced by the method have optimal ligate binding without overly dense binding sites or stearic effects. Azlactone-functional supports, especially porous supports such as porous particles, benefit from control of ligand density. A linear relationship exists between the molar ratio of ligand and quencher and the coupled ligand density. Optionally, hydrophilicity of the derivatized support can be determined by selection of quencher relative to selection of ligand.

17 Claims, No Drawings

METHOD OF CONTROLLING DENSITY OF LIGAND COUPLED ONTO SUPPORTS AND PRODUCTS PRODUCED THEREFROM

FIELD OF THE INVENTION

This invention concerns a method of controlling the density of ligands covalently coupled onto a support and the products produced from such method.

BACKGROUND OF THE INVENTION

Derivatization of polymeric supports is central to the preparation of various types of diagnostic and chromatographic media. Coupling of ligands to supports, i.e. covalent attachment of specific molecules or functional groups, is necessary to impart to those supports the ability to effect the separation, identification, and/or purification of molecules of interest. Prior art techniques for controlling the concentration or density of ligands on a polymeric support generally fall into one of four categories or combinations thereof:

a) Manipulation of reaction conditions which "activate" the matrix, i.e. which introduce a reactive group which can couple to the ligand. This often involves varying the concentration of "activating reagents", reaction time, reaction temperature, pH, or combinations of these variables.

b) Manipulation of reaction conditions during coupling of the ligand to the support. This may involve varying the concentration and/or the total amount of ligand the support is challenged with, ionic strength of the coupling buffer, and type of salt in the coupling buffer as well as the variables of time, temperature, pH, etc., mentioned above.

c) Manipulation of the amount of reactive or "activatable" functional group incorporated into the polymer support by varying polymer composition at the time of its formation, i.e. during the polymerization.

d) Manipulation of the amount of ligand incorporated into the polymer by preparation of a polymerizable ligand monomer and varying the concentration of this monomer in the monomer feed during polymerization.

For the most part, the above techniques for controlling ligand concentration on polymeric supports are quite difficult to apply in a practical and reproducible manner, primarily because of the large number of variables which must be simultaneously controlled. This is especially true of the first two techniques, in which the efficiencies of the reactions (i.e., extent of desired reaction as opposed to competing side reactions) is strongly influenced by reaction conditions. Technique "c" seems to offer some degree of control, although one must subsequently apply the techniques of "a" and/or "b" in a second step to couple the ligand. Technique "d" would appear to provide exact control of ligand density until one realizes that many of the ligands which are useful for diagnostic applications and chromatographic separations contain functional groups which are incompatible with conditions necessary for formation of the desired polymer (e.g., they are unstable under the contemplated polymerization conditions, or they interfere with the polymerization reaction, such as by inhibition of polymerization).

Successful ligand coupling is based on two factors: quantity immobilized and quality of immobilization. Quantity immobilized, expressed as weight of ligand per unit volume of support, is an indicator of the amount of ligand coupled regardless of the quality of that immobilization. Quality of immobilization is an indicator of the relationship between the amount of ligand coupled onto a support and the ability

of that ligand to maintain its binding interactions with a ligate such that it retains usefulness for chromatographic or diagnostic activity. Optimizing that activity is desirable and can be accomplished by manipulating either quantity or quality of immobilization, or both, depending on the desired use application. However, there must be enough ligand density to achieve practical utility.

While a support can couple at maximum ligand density, the binding of ligate to that ligand during chromatography can be hindered or altered by a number of factors including multiple-site binding of the ligand to the support (especially relevant with high molecular weight ligands) and steric hindrance due to proximity of adjacent ligands. Thus, overly-dense ligand coupling to a support is wasteful of the ligand and unnecessary or deleterious to the binding activity, especially for such applications as affinity chromatography and diagnostics. In these instances, the optimal condition in ligand coupling would be the achievement of maximum possible density of ligand coupled while maintaining maximum chromatographic or diagnostic activity with respect to ligate binding to said ligand. That results in optimal ligate binding or functional efficiency of the coupled ligand on the support.

For other chromatographic separations, such as for hydrophobic interaction or reverse phase, chiral, and ion exchange chromatography, high quantity of immobilization may lead to too strong a binding interaction, thus leading to difficult elutions or losses in resolution. In these cases, the ability to decrease ligand density or alter its distribution will improve chromatographic performance in terms of selectivity, resolution, and recovery of ligate.

Many ligand candidates are large molecules such as proteins and enzymes that have specific conformations necessary to retain biological activity. Recently attempts have been made to overcome the limitations in the prior art with respect to coupling of these high molecular weight ligands. In the case of antibody binding of antigen, low antigen binding efficiencies have been attributed to the concerted actions of surface density of antibody, multi-point attachment of antibody to porous supports, and undesirably restrictive conformations imposed by covalent attachment. See Velander et at., "The Use of Fab-Masking Antigens to Enhance the Activity of Immobilized Antibodies", *Biotechnology and Bioengineering*, Vol. 39, 1013–1023 (1992) which discloses how enhanced functional efficiency was achieved when the Fab portion of a monoclonal antibody was masked with synthetic antigens prior to covalent immobilization of the antibody on the support, followed by unmasking.

U.S. Pat. No. 5,200,471 (Coleman et al.) describes a method for the covalent immobilization of biomolecules on azlactone-functional polymeric supports in the presence of polyanionic salt and a buffered aqueous solution. Preferably, the immobilization also occurs in the presence of an azlactone quencher, i.e. another molecule which will compete with the protein ligand for the azlactone groups through which covalent coupling occurs. Incorporation of the quencher in this procedure in concentrations in the range of 4 to 6 orders of magnitude higher than the concentration of the biomolecule seems to have some effect on controlling the density of the coupled protein ligand. However, its major effect appears to be manifest in improvements in bound specific biological activity of the ligand.

Other attempts to control ligand density and/or distribution of biomolecules are described in U.S. patent application Ser. No. 08/038,645 (Velander et al.) and in references therein.

Using a very different approach, U.S. Pat. No. 4,968,742 (Lewis et al.) teaches a two step process for control of ligand density by controlling the number of active sites for ligand coupling. Step one of the complicated process involves reacting the non-activated polymeric material with a predetermined ratio of excess amounts of an "activating" agent and a "blocking" agent. In a second step, the ligand without any other agent competing for coupling sites is covalently coupled to a functional group on the "activating" agent. While this method again offers some degree of control over the density of ligands, it is still somewhat limited. The method indeed only allows control over the introduction of the "activating" agent, with the, subsequent coupling of the ligand being subject to the deficiencies discussed above.

SUMMARY OF THE INVENTION

Controlling ligand density on a support is a problem unsolved sufficiently in the prior art for low molecular weight ligands without complicated multi-step procedures. The deficiencies of the prior art are overcome by the present invention.

In the present invention, ligand is covalently coupled in the presence of a suitable quencher in a single step.

The method solves the problem of controlling ligand density by reacting the polymer support with a mixture of the ligand and quencher, the molar ratio of ligand to quencher in the mixture being essentially the ratio desired to be coupled to the support.

The method avoids excessive usage of quencher relative to the amount of ligand to be coupled and avoids the requirement of coupling in a buffered aqueous solution having a high concentration of polyanionic salts therein, as is required in U.S. Pat. No. 5,200,471.

The method of the present invention is particularly advantageous for the coupling of small molecule ligands (i.e. low molecular weight ligands below about 1000 atomic mass units) for the purposes of preparing ion exchange, hydrophobic interaction or reverse phase, chiral, and affinity chromatography supports.

Preferred supports for coupling are azlactone-functional supports.

Preferred ligands and quenchers comprise primary and secondary amine-functional molecules.

Products produced from the method of the present invention are advantageous in that ligand density of low molecular weight ligands is controlled for optimal ligate binding with minimal unnecessary use of ligand for coupling to the support.

A feature of the present invention is comparatively unrestricted reaction conditions for covalent coupling of ligand to the support. That is, relatively broad ranges of reactions conditions such as concentration, time, temperature, etc., can be used without significantly affecting the outcome of the coupling reaction.

Another feature of the present invention is ability to control the ligand density without first requiring a step to activate or deactivate reaction sites on the support.

An advantage of the present invention is the ease with which ligand density on the support can be manipulated in a single step coupling reaction. This can be a powerful tool for optimization of a chromatographic separation by optimizing ligate binding and elution.

In describing the present invention:

"Ligand" means a molecule or species covalently immobilized on a support and which comprises functional group(s) which interact with other species in solution (ligates) by ionic, hydrophobic, hydrogen-bonding, etc., type interactions.

"Ligate" means a molecule or species which can interact, in a reversible manner, with a support comprising an immobilized ligand.

"Coupling" refers to immobilizing, by means of a covalent chemical bond, a ligand to a support; this is generally an irreversible reaction.

"Binding" refers to the interaction of the ligate with the coupled ligand; this is generally a reversible reaction which can be disrupted by changes in pH, ionic strength, etc., of the solution.

"Directly covalently reactive" and words of similar import refer to supports having functional groups associated therewith that do not require further reaction in order to couple ligands to a support.

"Density" refers to the concentration of ligand on a support and is generally described in terms of either weight or molar equivalents of ligand per unit weight or volume of support.

"Resolution" means the degree of separation of two adjacent eluting solutes (ligates) in a chromatographic run.

"Selectivity" is another term describing separation of solutes in a chromatographic run and is used herein to refer to the overall chromatographic profile (retention time, separation, elution order, etc.) for a series of ligates.

"Recovery" refers to the amount of purified ligate able to be eluted from a chromatographic column relative to the amount available in the originally applied feed.

EMBODIMENTS OF THE INVENTION

Supports

Acceptable supports for use in the present invention can vary widely within the scope of the invention. A support can be porous or nonporous, depending on preferred final use. A support can be continuous or non-continuous depending on ultimate desired usage. A support can be made of a variety of materials, including supports made of ceramic, glassy, metallic, or polymeric materials or combinations of materials. A support can be flexible or inflexible depending on ultimate desired usage.

Preferred supports include polymeric supports, such as particulate or beaded supports, woven and nonwoven webs (such as fibrous webs), microporous fibers, and microporous membranes.

Woven and nonwoven webs having either regular or irregular physical configurations of surfaces are useful as supports. Fibrous webs are particularly desired because such webs provide large surface areas, with nonwoven fibrous webs being preferred due to ease of manufacture, low material cost, and allowance for variation in fiber texture and fiber density. A wide variety of fiber diameters, e.g., 0.05 to 50 micrometers, can be used. Web thickness can be varied widely to fit the application, e.g., 0.2 micrometer to 100 cm thick or more. Fibrous webs can be prepared by methods known in the art, or by modifications of methods known in the art.

Pre-existing, polymeric supports can also include microporous membranes, fibers, hollow fibers, or tubes, all of which are known in the art.

Ceramic supports, glass supports, and metallic supports are all known in the art and are commercially available or can be prepared by a variety of known techniques.

Generally, supports useful in the present invention are described in U.S. patent application Ser. Nos. 08/038,645 (Velander et al.); 07/896,107 (Gagnon et al.); and 07/776,601 (Rasmussen et at.), the disclosures of which are incorporated by reference herein.

Preferably, the supports for use in the present invention are porous supports and include those commercially available for chromatography techniques. The porous support can be any porous solid, whether natural or synthetic, organic or inorganic, having a porous structure and which is insoluble in water or aqueous solutions. Suitable solids with a porous structure have pores of a diameter of at least 1.0 nanometers (nm) and a pore volume of over 0.1 cm^3/g. Preferably, the pore diameter is at least 30 nm because larger pores will be less restrictive to diffusion. Preferably, the pore volume is at least 0.5 cm^3/g for greater potential capacity due to greater surface area surrounding the pores. Preferred porous supports include particulate or beaded supports.

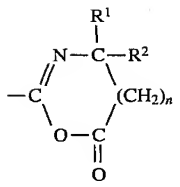
In order to be useful for the purposes of the invention, the support must be a reactive support; that is, it must comprise a reactive functional group which can be used for coupling to the desired ligand. That reactive functional group should be capable of undergoing rapid, direct covalent coupling with the desired ligand to form derivatized supports. The covalently reactive functional groups which are useful for the purposes of the invention can be classified in general as electrophiles. Reaction with a nucleophile (e.g. amine, alcohol, or mercaptan) produces a covalent chemical bond either by an addition reaction or by a displacement or substitution type reaction (in which a byproduct molecule is released). Addition type reactions are preferred. While it is possible to use the various "activating chemistries" well known in the art to introduce reactive functional groups useful, at least to a certain extent, for the purposes of this invention, this approach is subject to the problems described earlier which make it difficult to achieve reproducible results. Thus, it is preferred to utilize supports, especially commercially available supports, which can be obtained with a reproducible level of reactive functionality.

A number of useful particles and membranes are commercially available which contain reactive functional groups. These useful reactive functional groups include N-hydroxysuccinimide esters, sulfonyl esters, iodoacetyl groups, aldehydes, imidazolyl carbamates, and cyanogen bromide activated supports.

Generally, these reactive functional groups are described in U.S. patent application Ser. Nos. 08/038,645 (Velander et al.) and 07/776,601 (Rasmussen et at.), the disclosures of which are incorporated by reference herein.

Although these functional groups may be used in the method described herein, they are not preferred because they are subject to a number of problems including thermal instabilities and secondary reactions or hydrolysis reactions which can compete with the desired coupling reaction.

Particularly preferred as reactive supports useful in the present invention are supports having azlactone-functional groups on internal and/or external surfaces of such supports. Thus, such reactive supports have an azlactone-functional group of Formula I:



I

wherein:

R^1 and R^2 independently can be an alkyl group having 1 to 14 carbon atoms, a cycloalkyl group having 3 to 14 carbon atoms, an aryl group having 5 to 12 ring atoms, an arenyl group having 6 to 26 carbon atoms and 0 to 3 S, N, and nonperoxidic O heteroatoms, or R^1 and R^2 taken together with the carbon to which they are joined can form a carbocyclic ring containing 4 to 12 ring atoms, and

n is an integer 0 or 1.

Azlactone-functional reactive supports are particularly preferred in the present invention because such supports rapidly and directly covalently couple ligands better and with fewer side reactions (e.g., hydrolysis) than other commercially available supports reactive functional groups. Further, such azlactone-functional groups are quite stable prior to covalent coupling with a ligand. Further, covalent coupling of a ligand with an azlactone-functional group causes no displacement of a hazardous byproduct molecule, which minimizes problems of purification of the article after covalent coupling of the ligand.

Also, azlactone,-functional supports are known to possess high covalent coupling capacities with nucleophilic ligands. Further, such high covalent coupling capacities provides the ability to control ligand density over a wide range according to the present invention. Thus, an azlactone-functional reactive particle is particularly preferred for use in the present invention.

Azlactone-functional polymeric particles can be made, for example, by copolymerization of a (meth)acryloylamino acid with a variety of other free radically polymerizable comonomers followed by reaction with a cyclizing agent, as described in U.S. Pat. Nos. 4,737,560 and 4,871,824, which are incorporated herein by reference, or by copolymerization of an alkenyl azlactone with other comonomers as described in U.S. Pat. No. 5,292,840, which is incorporated herein by reference. Azlactone-functional particles can also be prepared by solution coating an azlactone-functional polymer onto an organic or inorganic particle or support, also as described in U.S. Pat. No. 5,292,840.

Azlactone-functional particles can also be produced by graft polymerization onto base supports which contain aliphatic hydroxyl groups on their surfaces as described in European Patent Disclosure 0,565,978 A1, the disclosures; of which are incorporated by reference.

Azlactone-functional reactive particles can also be made from azlactone graft copolymers which are disclosed in U.S. Pat. Nos. 5,013,795 and 5,262,484, the disclosures of which are incorporated by reference.

Size of azlactone-functional particles can be from about 0.1 to 1,000 micrometers and preferably from 0.5 to 250 micrometers. Dry azlactone-functional particles can have an average pore size ranging from about 1 to about 300 nanometers and preferably from 5 to about 200 nanometers. Azlactone-functional particles can have an average pore volume of at least 1.0 cm^3/g of particle. In a particle having a size of 50–80 micrometers, a pore volume of at least 1.2 cm^3/g provides a pore volume of about 60% of the particle volume. In the same particle, the surface area is at least 50 m^2/g. Thus, there is substantial surface area within an

azlactone-functional particle available for covalent immobilization according to the present invention.

Most preferably, porous supports useful for the present invention are Emphaze™ brand porous azlactone-functional activated affinity chromatography beads commercially available from Minnesota Mining and Manufacturing Company of St. Paul, Minn.

Other azlactone,-functional supports are also useful in the present invention. Pre-existing supports, such as fibrous supports and microporous membranes, can be rendered azlactone-functional using the disclosure of copending and coassigned U.S. patent application Ser. No. 07/896,107, the disclosure of which is incorporated by reference herein. Multi-functional azlactone supports disclosed in U.S. Pat. No. 5,292,514 (Capecchi et al.), the disclosure of which is incorporated by reference, are also useful as supports for the present invention. Azlactone-functional particles can also be incorporated into continuous porous matrices as disclosed in copending and coassigned U.S. patent application Ser. No. 07/776,601, the disclosure of which is incorporated by reference herein.

Ligands for Covalent Immobilization

As stated above, reactive functional groups on porous supports are desirably electrophiles. Thus, for direct covalent immobilization, ligands useful in the present invention contain nucleophilic groups for coupling to the electrophilic groups of the support. Nonlimiting examples of ligand nucleophilic functional groups include primary and secondary amines, alcohols, and mercaptans. Of these, amine-functional ligands are especially preferred.

Ligands useful for the preparation of derivatized supports can vary widely within the scope of the present invention. In order to be useful as a ligand, the molecule must contain some other functional group (or groups) useful in the contemplated end use application. Preferably, a ligand is chosen based upon the desired use of the derivatized support. End uses include applications as chromatographic supports, diagnostic reagents, metal ion complexation or removal, etc. For example, carboxylic or sulfonic acid containing ligands are useful for the preparation of cation exchange supports, while amine containing ligands (primary, secondary, or tertiary amines) are useful for the preparation of anion exchange supports. Ligands containing aromatic or aliphatic groups are useful for the preparation of supports for hydrophobic interaction or reverse phase chromatography. Ligands containing functional groups which undergo specific binding interaction with biological molecules are useful for the preparation of affinity supports or diagnostic reagents.

Once ligands are coupled according to methods of the present invention, such ligands are available for biological or chemical interaction with an enhanced functional efficiency, such as adsorbing, complexing, catalysis, or reagent end use.

Derivatized supports are useful as adsorbents, complexing agents, catalysts, reagents, and as chromatographic articles.

Ligands useful in the present invention generally have a low molecular weight, i.e., below about 1000 atomic mass units. Presently preferred azlactone-functional groups will undergo nucleophilic attack by amines, thiols, and alcohols. Thus, ligands having at least one amine, thiol, or alcohol group thereon are candidates for covalent immobilization on an azlactone-functional support. For reasons of increased stability of the linkage formed upon coupling, amine-functional ligands are preferred for the purposes of the present invention. Nonlimiting examples of such ligand materials include amine-containing compounds such as amino acids, diamines, aromatic amines, primary and secondary aliphatic amines, and hydrazines and hydrazides.

Quencher

The type of quencher can vary according to the nature of the ligand to be coupled to the support. However, the quencher must have reactivity with the same activated site on the support as does the ligand. The kinetics of reaction (as influenced by pH, ligand concentration, reaction time, and temperature, among other factors) between the support and the ligand determine the amount and type of quencher to be used.

Preferably, the quencher will have essentially the same kinetics of reaction with the support as does the ligand. Thus, if the ligand has a primary amine nucleophile for coupling to the support, the quencher will also preferably comprise a primary amine nucleophile. When this condition holds, the density of coupled ligand is essentially controlled by the molar ratio of the ligand and quencher in the coupling solution. However, as is well known to one skilled in the art, the ligand and the quencher will not exhibit exactly identical kinetics of reaction with the support. Substituents on either the ligand or the quencher will influence the nucleophilicity of the respective reactants as well as contribute to the steric environment around the nucleophilic groups. Both of these effects may influence the overall kinetics of the coupling reaction.

While not being limited to a particular theory, a quencher competes for the reactive sites on a porous support where ligand would otherwise couple. The reduction in the number of reactive sites can limit the possibility that ligand couples in a manner that creates overly dense binding sites or otherwise alters the conformation of biologically active ligates bound to such sites, reducing or eliminating binding activity. Unexpectedly, it is believed that a quencher optimizes ligate binding by providing a sparcity of reactive sites without eliminating too many reactive sites for binding. This also tends to result in a more uniform or effective distribution of coupled ligand.

Optionally, the choice of quencher may be made so as to not only control ligand density but also to influence the resultant hydrophilicity or hydrophobicity of the derivatized support matrix. Use of a very hydrophilic quencher (ammonia, for example) will result in the formation of a much more hydrophilic environment surrounding the coupled ligand than that which will result from use of a more hydrophobic quencher (such as ethanolamine or ethylamine, for example). This result may be very important when one wants to maximize specific interactions of the ligand with the desired ligate, as for example in affinity or diagnostic applications. Alternatively, use of a more hydrophobic quencher may promote weaker, secondary interactions of ligates with the surrounding matrix. Such effects are useful in control of chromatographic selectivity and resolution. Thus, the ability to control both ligand density and hydrophilic/hydrophobic effects can be very powerful for optimization of chromatographic performance.

When preferred azlactone-functional porous supports are used, quenchers are azlactone quenchers. Suitable azlactone quenchers are also identified in U.S. Pat. No. 5,200,471 (Coleman et al.), the disclosure of which is incorporated by reference.

Nonlimiting examples of azlactone quenchers for use include ethanolamine, hydroxylamine, methylamine, aniline, ethylamine, ammonium hydroxide, ammonium sulfate, butylamine, glycinamide, TRIS (trishydroxymethylaminomethane), glycerylamine, glucosamine, acethydrazide, and combinations thereof.

The concentration of azlactone quencher in the reaction medium can range from about 0.01M to about 10M. Desirably, the range may be between about 0.05M to about 2M. When ethanolamine serves as azlactone quencher, the concentration may range from about 0.1M to about 1M. The presently preferred concentration of ethanolamine as azlactone quencher is about 0.1M to about 0.5M.

Method of Competing Covalent Immobilization

The method of the present invention involves a single step in which the desired ligand and the quencher compete for the same activated sites on the support.

The reaction conditions for competing covalent immobilization of desired ligand and quencher are nonspecific and variable without otherwise affecting the competition of ligand and quencher for the same activated sites on the support.

The reaction solvent can be aqueous, organic, or mixed but should be capable of dissolving or sufficiently dispersing both ligand and quencher for true competition. The medium can be buffered or non-buffered but should not favor ligand over quencher, or vice versa. The medium can have a low or high concentration of polyanionic salts, as the presence of these salts generally does not affect the coupling.

The reaction temperature is preferably ambient but can vary from about 0° C. to about the boiling point of the solvent. The reaction pressure can be ambient.

Concentrations of ligand and quencher in the reaction medium should be comparable, i.e., within two orders of magnitude and preferably within one order of magnitude. Most preferably, the molar ratio of ligand concentration to quencher concentration can range from about 20:1 to about 1:20. In addition, the combined concentrations of ligand and quencher (reactants) should be such that there is an excess on an equivalent basis relative to the total quantity of reactive functional groups on the support. Although satisfactory results can sometimes be obtained with an equivalent ratio of reactants to functional groups of about 2:1, best results are obtained with a ratio of at least 10:1, preferably of about 20:1, and most preferably of about 100:1.

The reaction solution usually does not require buffering agents, especially when the nucleophile is a primary or secondary amine, but may include buffering agents if so desired. Buffering agents for aqueous media include acetate, phosphate, pyrophosphate, borate, and other salts known to those skilled in the art, such as those buffering agents disclosed in Good et al., *Biochemistry,* 5, (1966) p. 467 et seq. (the disclosure of which is incorporated by reference herein).

The concentration of buffering agents in aqueous media can range from about 10 mM to about 750 mM and desirably from about 50 mM to about 200 mM, inclusive, depending on the concentration of ligand and quencher chosen for coupling and the concentrations of other optional ingredients that can affect the ionic strength of the reaction solution or the solubilities of the ligand and quencher.

The duration of the competing immobilization should be sufficient in length to assure completed covalent coupling of ligand and quencher. From about 15 to about 240 minutes is usually sufficient, although longer reaction times can be used. Thus, the covalent immobilization even with competing quencher present is completed rapidly.

Coupling conditions are enhanced when pH of a reaction solution is changed to a pH within one pKa of the nucleophilic ligand, usually within a range from about 3 to about 12. A pH of this range provides coupling conditions that maximize the reaction of a nucleophilic group on a ligand or quencher with an electrophilic group on the surface of a support. However, the exact pH range chosen for coupling will depend upon the type of nucleophilic group present on the ligand and quencher. Thus, with primary or secondary aliphatic amine nucleophiles, a pH range of about 9 to about 12, preferably from about 11 to about 12 is used. With aromatic amines, a pH in the range of about 3 to about 6, preferably from about 4 to 5 is used. Also with aromatic, amines, a condensation agent such as a carbodiimide is preferably used. These conditions cause rapid and assured coupling of ligand and quencher to the support.

The rate of coupling is a function of the rate constant of coupling, the concentration of ligand, nucleophilicity of ligand and quencher, reactivity of the functional groups per unit area of the support, pH, and temperature, etc. In short, reaction kinetics for ligand and reaction kinetics for quencher should be understood and not employed to favor ligand over quencher, or vice versa, unless the amount of ligand density or hydrophilicity desired requires favoring a relatively unreactive quencher to compete with a very reactive ligand, or vice versa. However, when an excess of ligand plus quencher with respect to reactive functional groups is used as described above, these other reaction variables have relatively little influence over the outcome of the immobilization reaction.

As seen in the Examples below, unexpectedly, it has been found that with presently preferred azlactone-functional supports, a linear relationship generally obtains between the molar ratio of ligand and quencher and the final coupled ligand density. In addition, a linear relationship is generally observed between coupled ligand density and chromatographic capacity for protein ligates. These findings indicate the surprising absence of competing side reactions during immobilization and that all coupled ligand is capable of successful interaction with ligate, even at relatively high ligand densities. Thus, it is relatively, but unexpectedly easy in this situation to determine the molar ratio needed to obtain an optimized coupled ligand density or ligate capacity even when one does not have detailed kinetic information related to ligand and/or quencher reaction with the support. One can simply conduct two or three quick screening reactions (for example, 1-couple ligand in absence of quencher, 2-couple ligand and quencher at comparable concentrations, and 3-couple ligand in the presence of high concentration of quencher), evaluate either ligand density or chromatographic performance, plot the data, and use the linear relationship obtained to optimize ligand density. That linear relationship further militates toward the use of azlactone-functional supports in the method of the present invention.

As will be obvious to one skilled in the art, the slopes of the above described linear relationships will be affected to some degree by the relative kinetics of reaction of ligand versus quencher or by the relative strengths of the interactions of ligand with ligates. Therefore, each particular application may require separate optimization. In other words, the particular ligand density (and, optionally, hydrophilicity) which will give an optimal chromatographic purification of a particular protein from a particular feedstream may not by optimal for a different protein or a different feedstream.

However, using the method of the present invention greatly facilitates the necessary optimization process due to the fact that rigorous control over multiple reaction condition variables is not needed.

Usefulness of the Invention

The method of the present invention provides a process of optimizing ligand density on supports in such a fashion as to minimize surface crowding which can couple ligand without ligate binding benefit. Alternatively, the method provides a process for controlling ligand density in such a fashion as to minimize ligate binding which is too strong to allow easy elution. In addition, the method provides a process for optimizing ligand density in such a fashion as to optimize chromatographic performance. The derivatized support has coupled ligand with a controlled ligand density, optionally with a modified hydrophilicity or hydrophobicity, resulting in optimal ligate binding or optimal chromatographic resolution, selectivity, and recovery. In turn, the use of the derivatized support achieves a degree of control over selectivity of ligate binding and recovery of desired ligate that has not been known previously in the art.

Optimal ligand coupling can be expressed in terms of ligand density on the support using molar or mass concentration of ligand coupled per mass or volume of support, or molar or mass concentration of ligand coupled per unit area of support. Maximum ligand density obtainable for any support will be determined by the concentration of reactive functional groups originally present on the support. Ligand density can range from about 10% to about 99% of the original activated sites.

Azlactone-functional supports react with ligands to form adduct supports according to the reactions described in U.S. Pat. No. 5,292,840, the disclosure of which is incorporated by reference.

For a greater understanding of the scope of the invention, the following examples are provided.

Procedures

Cation Exchange Capacity: A 15 ml polypropylene disposable chromatography column was packed with 1 ml of derivatized bead support. The column was then equilibrated by washing with 10 ml of loading buffer, 10 mM MOPS (4-morpholinepropanesulfonic acid)/pH 7.5, and loaded with 10 ml of protein solution (chicken egg white lysozyme, pI 11.0, from Sigma Chemical Co., St. Louis, Mo., 10 mg/ml in the MOPS buffer). Unbound lysozyme was washed off with 30 ml of the MOPS buffer (three 10 ml fractions). Finally, bound protein was eluted with 15 ml of 1M NaCl in MOPS buffer. Protein recovered in the various fractions was determined by measuring the UV absorbance at 280 nm using a Hewlett-Packard Diode Array Spectrophotometer, Model 8452A, and compared to a standard curve prepared using pure lysozyme. The amount of protein recovered in the NaCl eluate was equated to the cation exchange capacity for the support.

Anion Exchange Capacity: The procedure used was identical to that above for cation exchange capacity except that the protein loaded was bovine serum albumin (BSA, Sigma Chemical Co.). Pure BSA was also used to construct the standard curve.

Ligand, quencher, and buffer solution preparation: All solutions were prepared, unless otherwise noted, in deionized water. When necessary, pH was adjusted with either 10M sodium hydroxide or 10N hydrochloric acid, as appropriate. All chemicals, buffer salts, etc., were commercial materials purchased from either Aldrich Chemical Co., Milwaukee, Wis., or Sigma Chemical Co., St. Louis, Mo., and used as received.

EXAMPLES 1–5

These examples illustrate the control of ligand density for the preparation of carboxyl-functional beads, useful for cation exchange chromatography. The following solutions were prepared:

Solution A (ligand solution)—1M aspartic acid, pH 9.0

Solution B (quencher solution)—3M Ethanolamine, pH 9.0

Mixtures of solutions A and B were then prepared; 5 ml of each mixture was added to 125 mg Emphaze Biosupport Medium AB 1 beads (azlactone-functional bead available from 3M Bioapplications, St. Paul, Minn., having an azlactone functionality of approximately 40–45 micromoles/ml of support), vortexed, and the resultant slurry allowed to react at room temperature with end-over-end agitation for two hours, filtered, washed with deionized water (3×10 ml), 1% HCl (10 ml), and finally with deionized water until the eluate was of neutral pH. The results of the derivatizations were assayed by measuring cation exchange capacity for lysozyme (Table 1).

TABLE 1

	Preparation of Carboxyl-functional Beads			
Example	Solution A (ml)	Solution B (ml)	Molar Ratio	IEX Capacity (mg/ml)
1	0	10	0:100	5.1
2	5	5	25:75	21.5
3	6	2	50:50	31.3
4	9	1	75:25	38.5
5	10	0	100:0	48.7

When ion exchange capacity is plotted versus the molar ratio of ligand (aspartic acid) to quencher (ethanolamine) concentration, a linear relationship is observed. This relationship can be used to prepare derivatized beads having IEX capacities different from those of the above examples simply by making the appropriate mixture of solutions A and B.

EXAMPLES 6–11

The following examples illustrate the control of ligand density in the preparation of amine-functional beads which are useful for anion exchange chromatography. The following solutions were prepared:

Solution A (ligand solution)—0.5M 1,6-hexanediamine, pH 11.0

Solution B (quencher solution)—1.0M ammonia, pH 11.0

Mixtures of solutions A and B were prepared (20 ml each), reacted with Emphaze AB 1 beads (1.00 g, corresponding to 8 ml when hydrated) for 2 hours at room temperature, then worked up by washing with deionized water until the eluate was of neutral pH. The quantity of immobilized amine ligand was analyzed as follows. The derivatized beads were washed successively (using a Buechner funnel, filter flask, and water aspirator setup) with 300 ml of deionized water, 300 ml of 0.1N HCl, and 300 ml 0.0001N HCl. Following the final wash, vacuum was maintained until the majority of the liquid had been removed and only a damp filter cake remained. The filter funnel with the filter cake was transferred to a clean 250 ml filter flask, and the ionically-bound

chloride ions were displaced by washing with two 50 ml portions of 10% (wt/wt) sodium sulfate. During each washing, the beads were suspended and allowed to stand in the solution for 1 minute prior to filtration. The combined sodium sulfate filtrates were mixed with 1 ml of 5% (wt/wt) potassium chromate, stirred vigorously with a magnetic stirrer, and titrated with 0.2500M silver nitrate to the faint red end point, noting the volume of titrant required. A sample of underivatized beads was used to determine a blank titration volume. The difference between the sample volume and the blank volume was used to calculate the amine content in micromoles/ml of bead support. Anion exchange capacity was determined using BSA (Table 2).

TABLE 2

	Preparation of Amine-functional Beads			
Ex.	Solution A (ml)	Solution B (ml)	Amine Content	IEX Capacity (mg/ml)
6	4	16	18.8	11.7
7	8	12	25.6	20.8
8	10	10	29.6	24.4
9	12	8	29.0	25.4
10	16	4	34.4	28.2
11	20	0	41.5	32.5

When anion exchange capacity is plotted versus amine content, again a linear correlation is observed which can be used to determine reaction conditions necessary to produce a derivatized bead having the desired ligand density (or corresponding IEX capacity).

EXAMPLE 12–15

The following solutions were prepared:

Solution A (ligand solution)—0.5M ethylenediamine

Solution B (quencher solution)—1.0M ammonia

Solution C (quencher solution)—1.0M ethanolamine

Mixtures of solutions A and either B or C were prepared and reacted with Emphaze AB 1 beads as in Examples 6–11, except that 1.25 grams of beads (corresponding to 10 ml when hydrated) were used for each reaction. Amine content (ligand density) was measured by a titration procedure similar to that used in Examples 6–11, and anion exchange capacity was measured similarly using BSA (Table 3).

TABLE 3

	Ethylenediamine-Derivatized Beads			
Ex.	Solution A (ml)	Solution (ml)	Amine Content	IEX Capacity (mg/ml)
12	20	0	39.1	18.4
13	10	(B) 10	23.7	10.2
14	4	(B) 16	14.1	0.7
15	10	(C) 10	27.2	7.7

The results again show that the level of ligand incorporation is easily controlled along with corresponding control of ion exchange capacity. In addition, the results indicate that choice of quencher may .exercise additional control over ligand density and capacity (compare Examples 13 and 15, where identity of quencher seems to change effectiveness of the ligand in terms of the expressed ion exchange capacity).

EXAMPLES 16–19

The following ligand solution was prepared:

Solution A—1.0M 2-diethylaminoethylamine

Quencher solutions B and C from Examples 12–15 were used to prepare mixtures for derivatization of Emphaze AB 1 beads as before (Table 4).

TABLE 4

	DEAE-Derivatized Beads			
Ex.	Solution A (ml)	Solution (ml)	Amine Content	IEX Capacity (mg/ml)
16a	20	0	30.2	15.4
16b	20	0	32.7	15.7
17	10	(B) 10	26.8	9.2
18	10	(C) 10	21.1	2.8
19	2	(C) 18	16.4	0.3

EXAMPLES 20–22

These examples illustrate control of ligand density when coupling an aromatic amine-functional ligand. The following solutions were prepared:

Solution A (ligand solution)—1.0M 4-aminobenzylamine in 0.1M MES (4-morpholineethanesulfonic acid), pH 4.0.

Solution B (quencher solution)—1.0M aniline in 0.1M MES, pH 4.0.

Mixtures of Solutions A and B were prepared and reacted with 1.25 g samples of Emphaze AB 1 beads at room temperature overnight in the presence of 300 mg EDC (N-ethyl-N'-dimethylaminopropylcarbodiimide). Workup and evaluation of ligand density (amine content) and anion exchange capacity for BSA was done as in previous examples (Table 5).

TABLE 5

	Benzylamine-Derivatized Beads			
Ex.	Solution A (ml)	Solution B (ml)	Amine Content	IEX Capacity (mg/ml)
20	20	0	36.8	35.8
21	10	10	26.8	9.3
22	4	16	3.3	0.8

These examples illustrate control of ligand density when the ligand being coupled is an aromatic amine and coupling must be assisted with a condensation agent, in this case a carbodiimide. This technique can also be used to control the ligand density of 4-aminobenzamidine, an affinity ligand for purification of trypsin and similar enzymes.

EXAMPLES 23–28

These Examples illustrate the relative ineffectiveness of polyanionic salts to influence the coupling of low molecular weight ligands. The following solutions were prepared:

Solution A (ligand solution)—1.0M 2-diethylaminoethylamine (DEAEA)

Solution B (ligand solution)—1.0M DEAEA in 1.0M sodium sulfate

Solution C (ligand solution)—0.5M ethylenediamine

Solution D (ligand solution)—0.5M ethylenediamine in 1.0M sodium sulfate

Solution E (quencher solution)—1.0M ethanolamine

Solution F (quencher solution)—1.0M ethanolamine in 1.0M sodium sulfate

Mixtures were prepared, reacted with Emphaze AB 1 beads, and evaluated as in previous examples (Table 6).

TABLE 6

Amine-derivatized Beads - Polyanionic Salt Effects

Ex.	Solution (ml)	Solution (ml)	Amine Content	IEX Capacity (mg/ml)
23	(A) 20	0	22.8	17.0
24	(B) 20	0	26.0	19.5
25	(A) 10	(E) 10	20.5	13.9
26	(B) 10	(F) 10	24.0	15.1
27	(C) 10	(E) 10	18.2	8.0
28	(D) 10	(F) 10	20.8	11.0

The differences in ligand content or IEX capacity obtained for coupling in the presence or absence of the polyanionic salt, sodium sulfate, are essentially within the variability observed for the test methods or for duplicate reactions (see, for example, Examples 16a and 16b).

COMPARATIVE EXAMPLES 1–3

These Examples illustrate the importance of conducting the derivatization reaction near the pK of the ligand and quencher. The following solutions were prepared:

Solution A (ligand solution)—0.5M 1,6-hexanediamine, pH 7.5

Solution B (ligand solution)—0.5M 1,6-hexanediamine in 1.0M sodium sulfate, pH 7.5

Solution C (quencher solution)—1.0M ethanolamine, pH 7.5

Solution D (quencher solution)—1.0M ethanolamine in 1.0M sodium sulfate, pH 7.5

Mixtures were prepared, reacted with Emphaze AB 1 beads, and evaluated as in previous examples (Table 7)

TABLE 7

Coupling of Aliphatic Ligands at pH 7.5

Comparative Example	Solution (ml)	Solution (ml)	Amine Content
1	A (20)	0	14.5
2	A (10)	C (10)	11.6
3	B (10)	D (10)	8.7

The results indicate that coupling at pH 7.5, in this case about 4 pH units below the pK of the ligand, results in a dramatic lowering in the density of coupled ligand, probably the result of competitive side reactions such as hydrolysis. Again, polyanionic salt has no enhancing effect.

EXAMPLES 29–30

These examples illustrate the control of ligand density for hydrophobic interaction chromatography. The following solutions were prepared:

Solution A (ligand solution)—0.5M benzylamine, pH 11.0

Solution B (quencher solution)—0.5M ethanolamine, ph 11.0

Mixtures of Solutions A and B were prepared and reacted with Emphaze beads as in previous examples. The samples were evaluated using a Waters Delta Prep 3000 chromatograph equipped with a Waters Lambda Max UV spectrophotometer and Maxima data acquisition software. Bead samples were packed in a Waters AP-1 column to a bed height of 1.3 cm (1.0 ml total volume). The binding buffer consisted of 1.5M ammonium sulfate, 50 mM sodium phosphate, pH 7.1; the elution buffer consisted of 50 mM sodium phosphate, pH 7.1; the sample protein solution consisted of chicken egg white lysozyme dissolved at a concentration of 5 mg/ml in the binding buffer. Five ml of sample protein solution was loaded onto the column at a rate of 1 ml/min. The column was then washed for 5 min with binding buffer at a rate of 1 ml/min, then for 10 min at 2 ml/min. The buffer was then changed to the elution buffer in a single step. Elution of the bound protein was monitored spectrophotometrically until the optical density of the eluted fractions returned to baseline. Example 29, derivitized with 20 ml of Solution A, was found to bind almost all of the applied protein, and the elution removed the bound protein in a sharp, symmetrical peak with a maximum optical density of about 0.7. Example 30, derivatized with a 50:50 mixture of Solutions A and B, showed a large amount of unbound protein in the flow through fractions, while the elution peak for the bound protein showed a maximum optical density of about 0.35.

EXAMPLES 31–33

These examples illustrate the attempt to control ligand density upon derivatization of oxirane (epoxy-functional) beads. The oxirane beads were prepared as follows:

A 1-liter creased, round bottomed flask equipped with a mechanical stirrer (stirring rate 450 rpm), nitrogen gas inlet, thermometer, and condenser was charged with toluene (188 ml), 0.133 g poly(isooctylacrylate-co-acryloylaminoisobutyramide), heptane (348 ml), and glycidylmethacrylate (0.72 ml). This mixture was stirred and heated to 35° C. while sparging with nitrogen. To the stirring mixture was added a solution of methylenebisacrylamide (13.3 g), isopropanol (90 ml), sodium persulfate (0.55 g), and deionized water (60 ml). After stirring for an additional 5 minutes, tetramethylethylenediamine (0.55 ml) was added to initiate polymerization. Polymerization was allowed to continue for a total of 4 hours, then the resultant beads were filtered, washed with acetone three times, and dried under vacuum overnight to produce oxirane beads having approximately 40 micromoles of epoxide functionality per milliliter of support. Beads from a 38–106 micrometer sieve cut were used in the derivatization experiments.

Solutions A and E from Examples 23–28 were used to derivatize 1.0 g samples of the above oxirane beads. Evaluation was conducted as in previous examples (Table 8).

TABLE 8

Derivatization of Oxirane Beads

Ex.	Solution A (ml)	Solution E (ml)	Amine Content	IEX Capacity (mg/ml)
31	20	0	39.1	26.7
32	10	10	33.6	25.4
33	4	16	32.3	22.9

These experiments show that a large change in the ratio of ligand to quencher has relatively little effect on the ligand level or chromatographic behavior of the derivatized beads.

While embodiments have been identified and exemplified, the following claims and their equivalents provide the scope of the present invention.

What is claimed is:

1. A method for controlling density of ligand coupled to an azlactone functional support, comprising the step of reacting ligand and quencher with activated sites on a

support under conditions sufficient to promote competition of ligand with quencher for the activated sites,

wherein the ligand is a molecule having a molecular weight of less than about 1,000 atomic mass units, and wherein the concentration of ligand and the concentration of quencher are within two orders of magnitude; wherein there is a linear relationship between the molar ratio of ligand concentration to quencher concentration and density of ligand coupled to the support; and wherein there is a linear relationship between molar ratio of ligand concentration to quencher concentration and chromatographic performance of the support.

2. The method of claim **1**, wherein the pH of the reaction ranges from about 3 to about 12 and within 4 pH units of the pK of the ligand.

3. The method of claim **1**, wherein the concentration of quencher ranges from 0.01M to 10M.

4. The method of claim **1**, wherein the support is porous.

5. The method of claim **4**, wherein the porous support is a porous particle.

6. The method of claim **1**, wherein the support is directly covalently reactive with the ligand and the quencher.

7. The method of claim **1**, wherein the ligand comprises amine-containing compounds, thiol-containing compounds, or alcohol-containing compounds and wherein the quencher comprises a compound that competes with the ligand for covalent reaction with azlactone on the support.

8. The method of claim **7**, wherein the ligand is an amine-containing compound and the quencher is an amine-containing compound.

9. The method of claim **8**, wherein the ligand is an amino acid.

10. The method of claim **9**, wherein the quencher is selected to determine hydrophilicity of the support.

11. A derivatized support, comprising a support having both ligand and quencher coupled to activated sites on the support according to the method of claim **1**.

12. The derivatized support, according to claim **11**, wherein the support is directly reactive with ligand and quencher without need for an intermediate activation step.

13. The derivatized support, according to claim **12**, wherein the ligand comprises an amine-containing compound, a thiol-containing compound, or an alcohol-containing compound.

14. The derivatized support, according to claim **13**, wherein the support is a porous particle.

15. The derivatized support, according to claim **13**, wherein the quencher is selected to determine hydrophilicity of the derivatized support.

16. A derivatized support, comprising an azlactone-functional support having activated sites covalently reacted with ligand and quencher, wherein the density of ligand coupled to the support ranges from about 10% to about 99% of the original activated sites, and wherein there is a linear relationship between molar ratio of ligand concentration to quencher concentration and the chromatographic performance of the support.

17. The derivatized support, according to claim **16**, wherein the ligand comprises an amine-containing compound, a thiol-containing compound, or an alcohol-containing compound.

* * * * *

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United States Patent [19]

Velander et al.

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[45] **Date of Patent: Nov. 2, 1999**

[54] **INSIDE-OUT CROSSLINKED AND COMMERCIAL-SCALE HYDROGELS, AND SUB-MACROMOLECULAR SELECTIVE PURIFICATION USING THE HYRDOGELS**

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[51] **Int. Cl.**[6] **B01D 15/08**; B01D 39/00; C02F 1/28

[52] **U.S. Cl.** **536/57**; 210/198.2; 210/502.1; 210/635; 210/656; 96/101; 502/404

[58] **Field of Search** 536/57; 210/198.2, 210/502.1, 635, 656; 96/101; 502/404

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[57] **ABSTRACT**

Relating to chromatographic processes and ion-exchange and affinity matrices, a spatial installation method for a bifunctional reagent that crosslinks and/or activates a polymer matrix is disclosed, with inside-outside installation of a bifunctional reagent on and within a polymer matrix. The polymer matrix is cellulose, agarose, or chitosan particles. The installation may be followed by inside-outside ligand attachment, by further reacting the matrix with a ligand or ionic group so that a higher concentration of ligand or ionic moiety occurs on the intra-particle volume than the outer matrix surface.

24 Claims, 37 Drawing Sheets

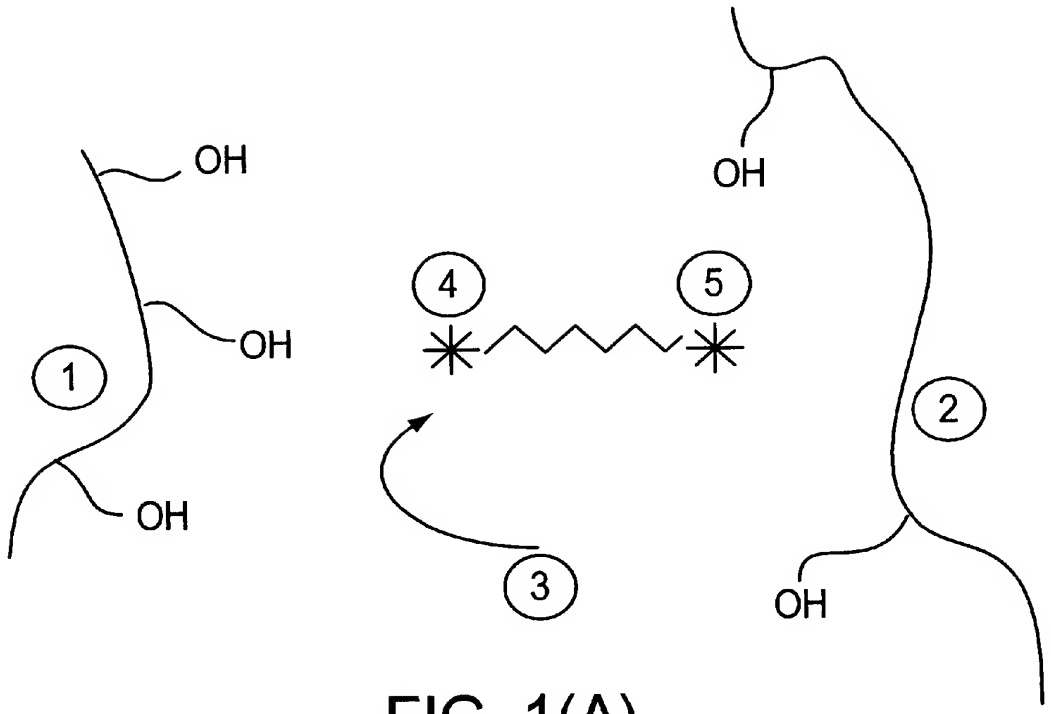


FIG. 1(A)

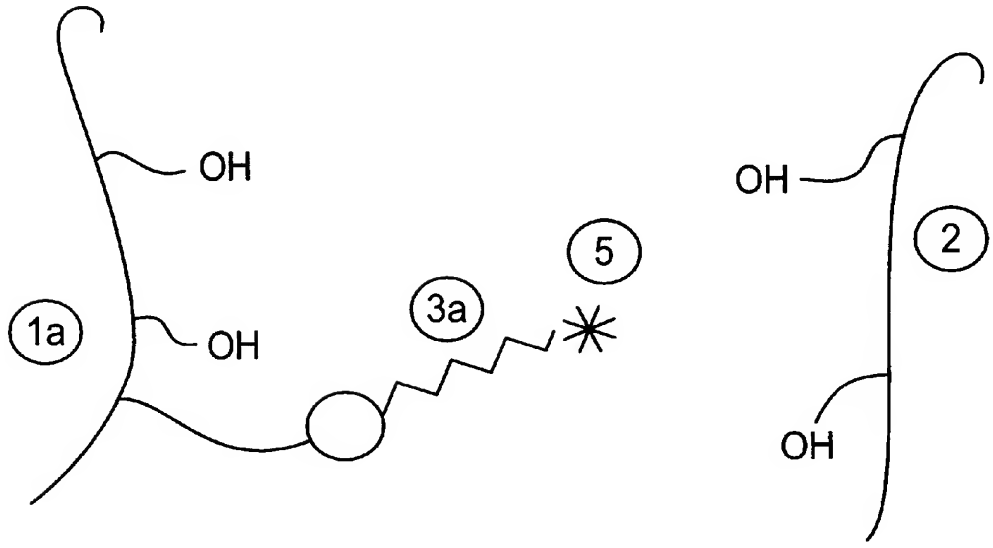


FIG. 1(B)

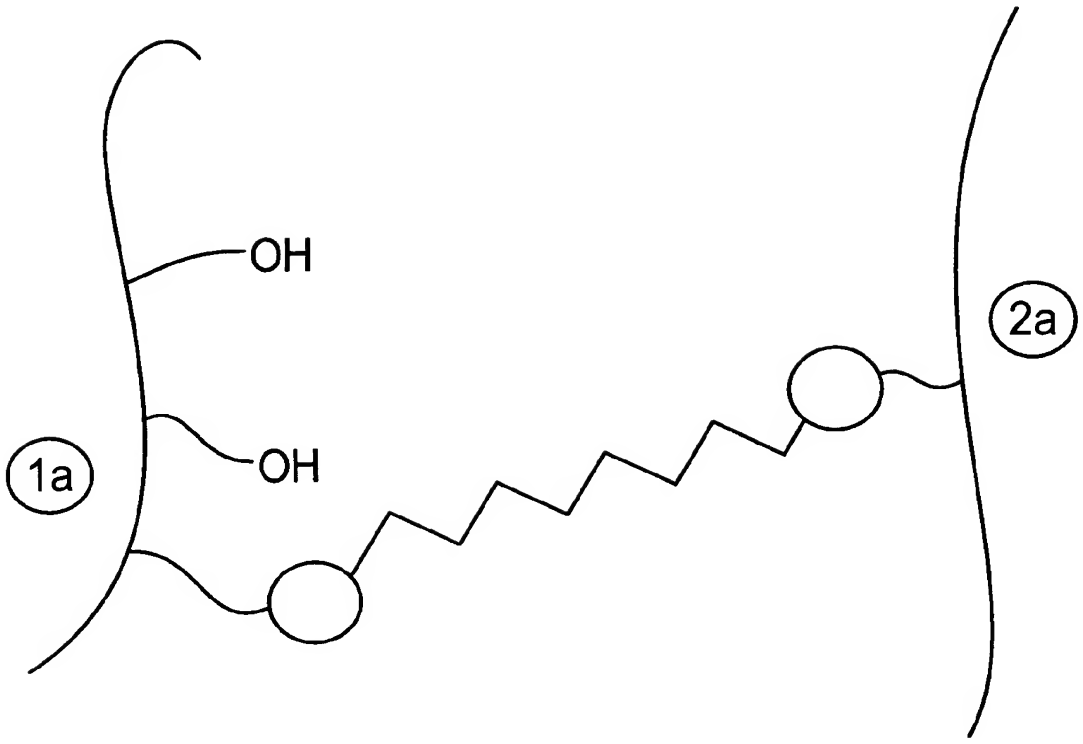
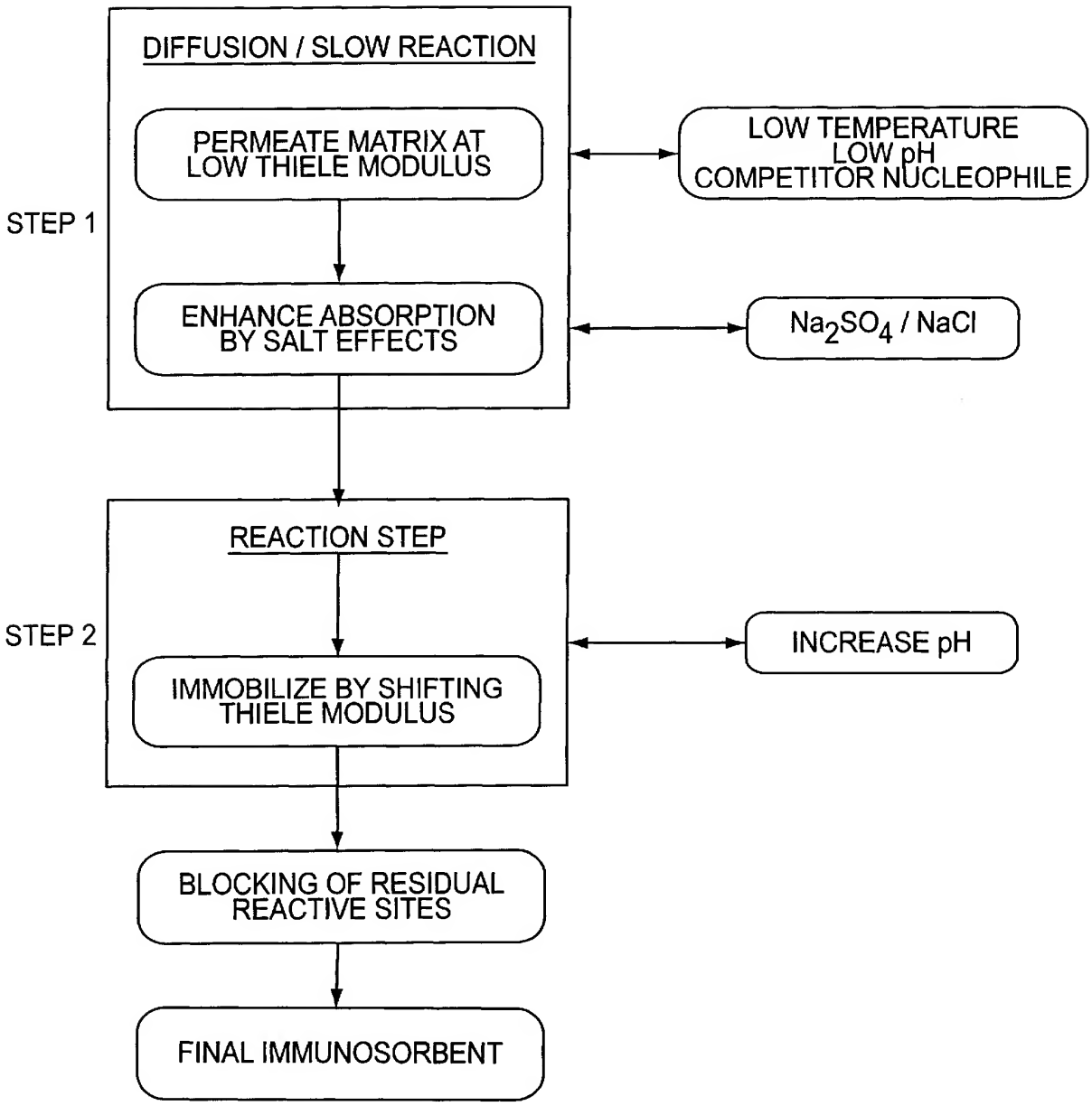


FIG. 1(C)

FIG. 2


DIFFUSION / SLOW REACTION
PERMEATE MATRIX AT LOW THIELE MODULUS
LOW TEMPERATURE LOW pH COMPETITOR NUCLEOPHILE
STEP 1
ENHANCE ABSORPTION BY SALT EFFECTS
Na_2SO_4 / NaCl
REACTION STEP
STEP 2
INCREASE pH
IMMOBILIZE BY SHIFTING THIELE MODULUS
BLOCKING OF RESIDUAL REACTIVE SITES
FINAL IMMUNOSORBENT

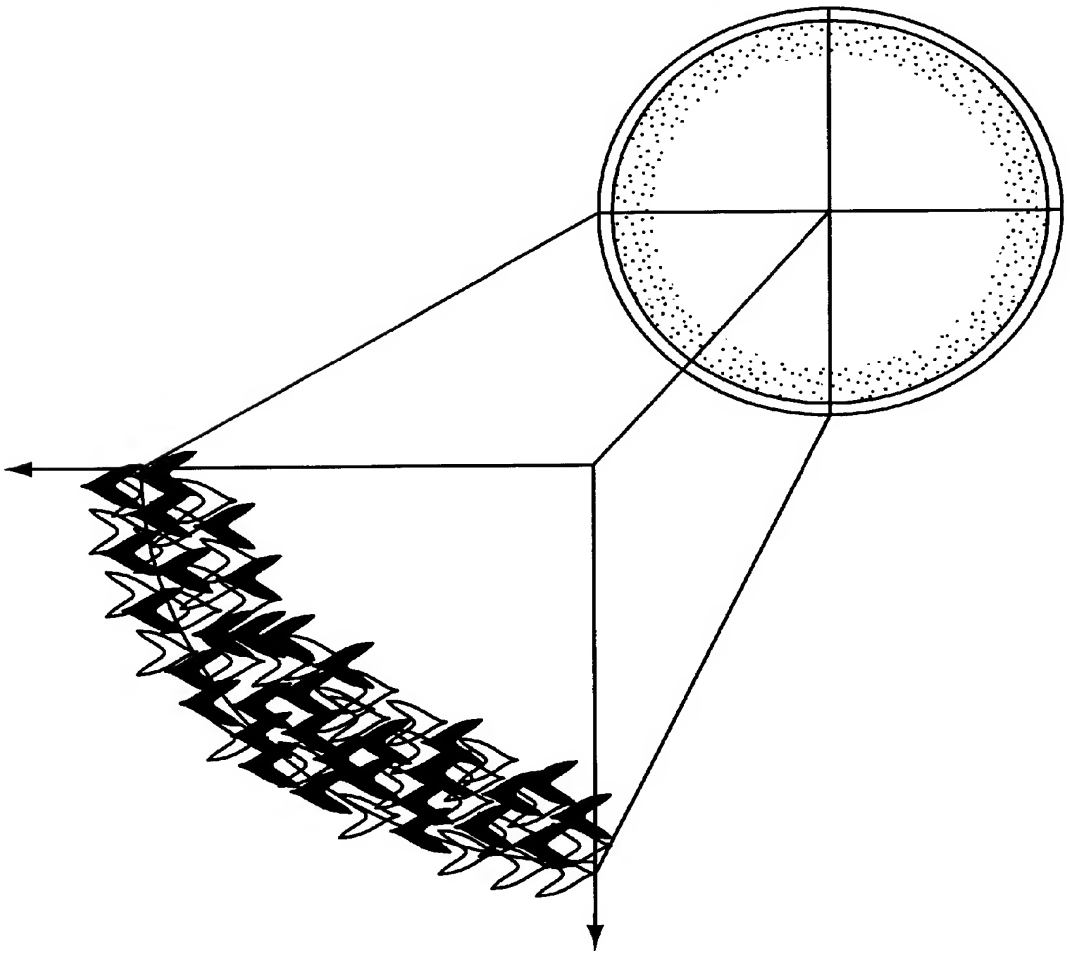


FIG. 3(A)

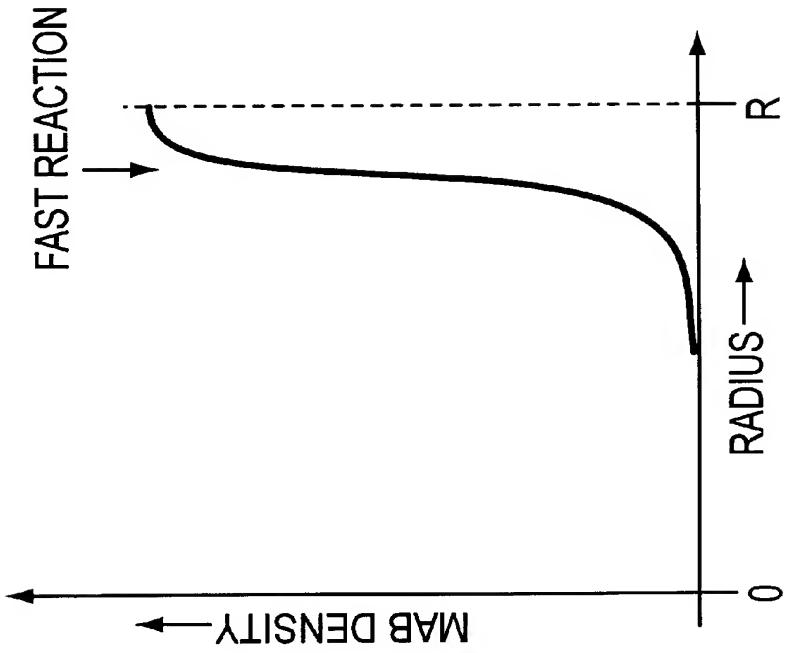


FIG. 3(B)

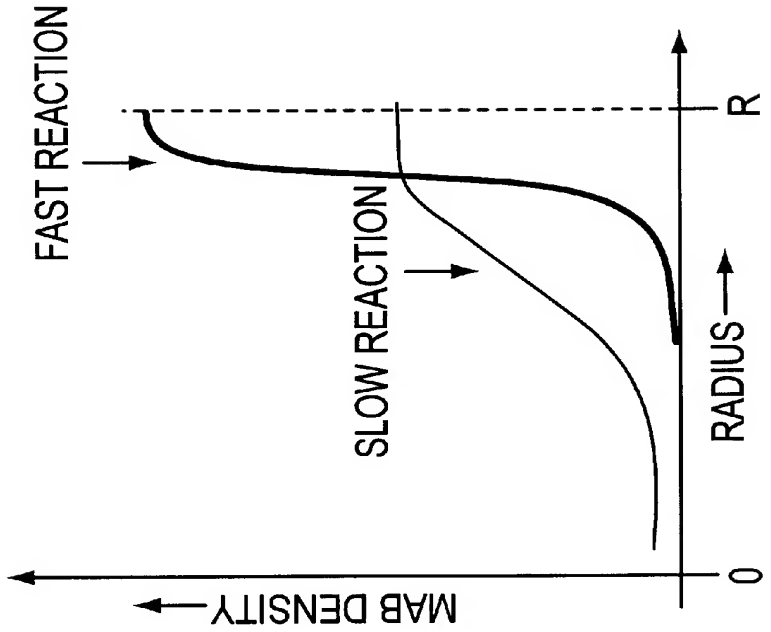


FIG. 4(B)

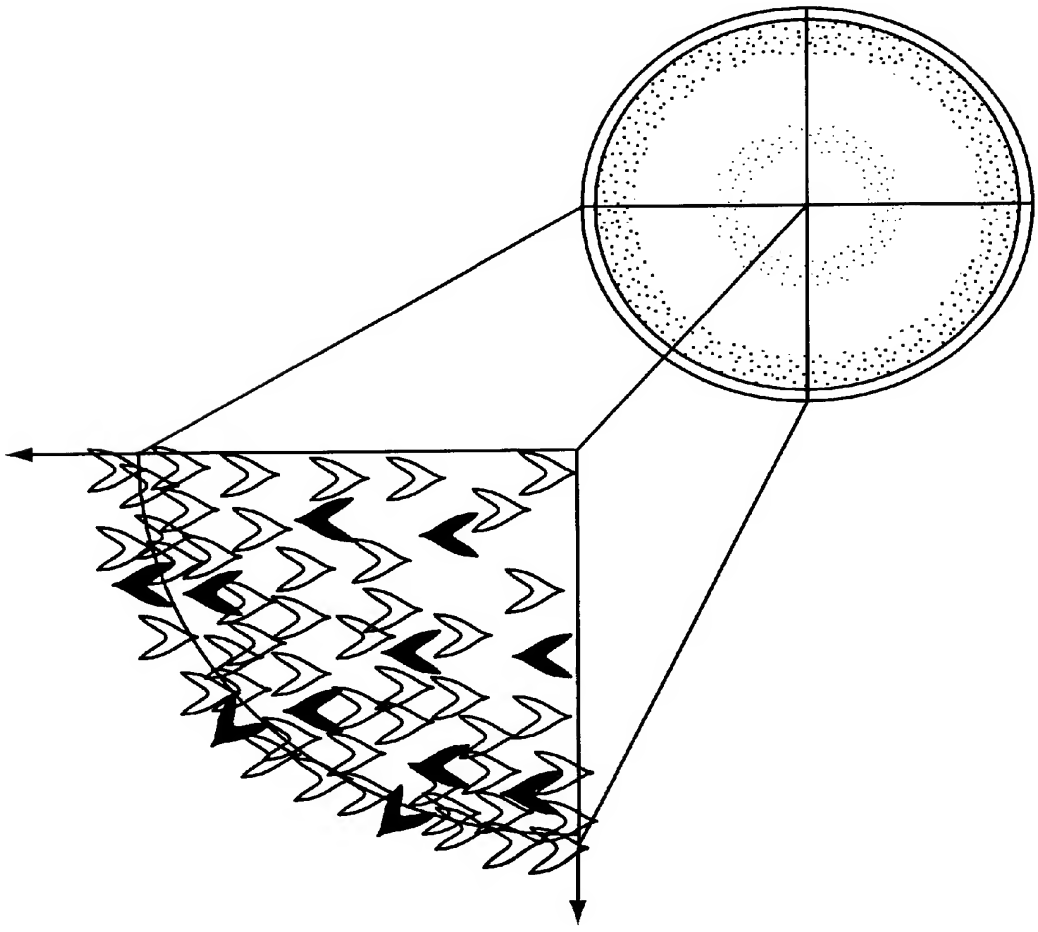


FIG. 4(A)

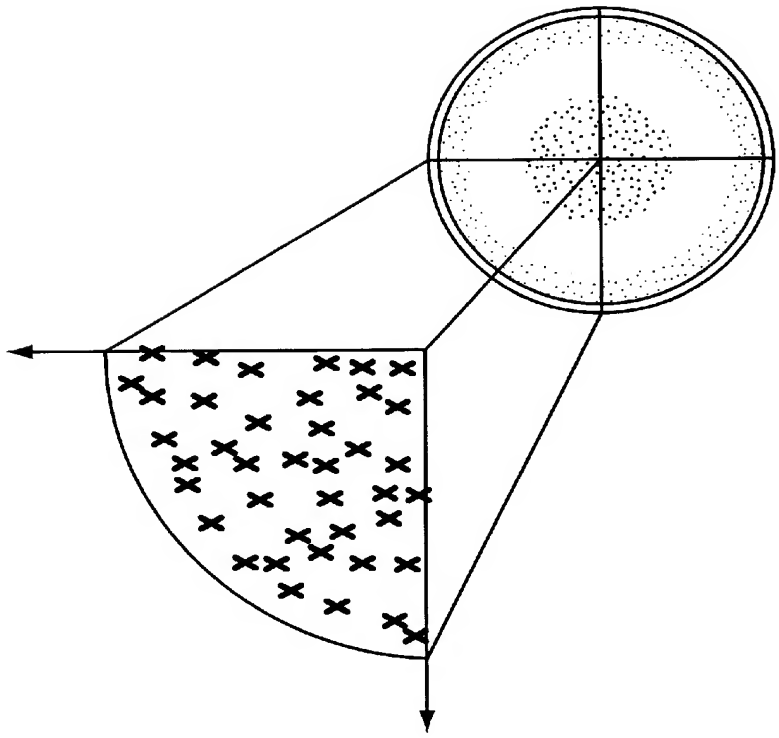


FIG. 5(B)

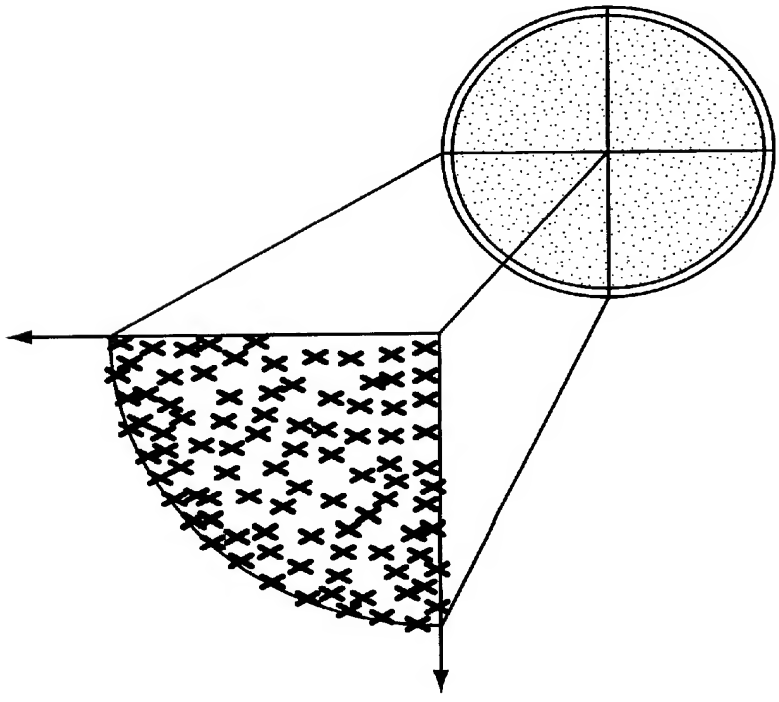


FIG. 5(A)

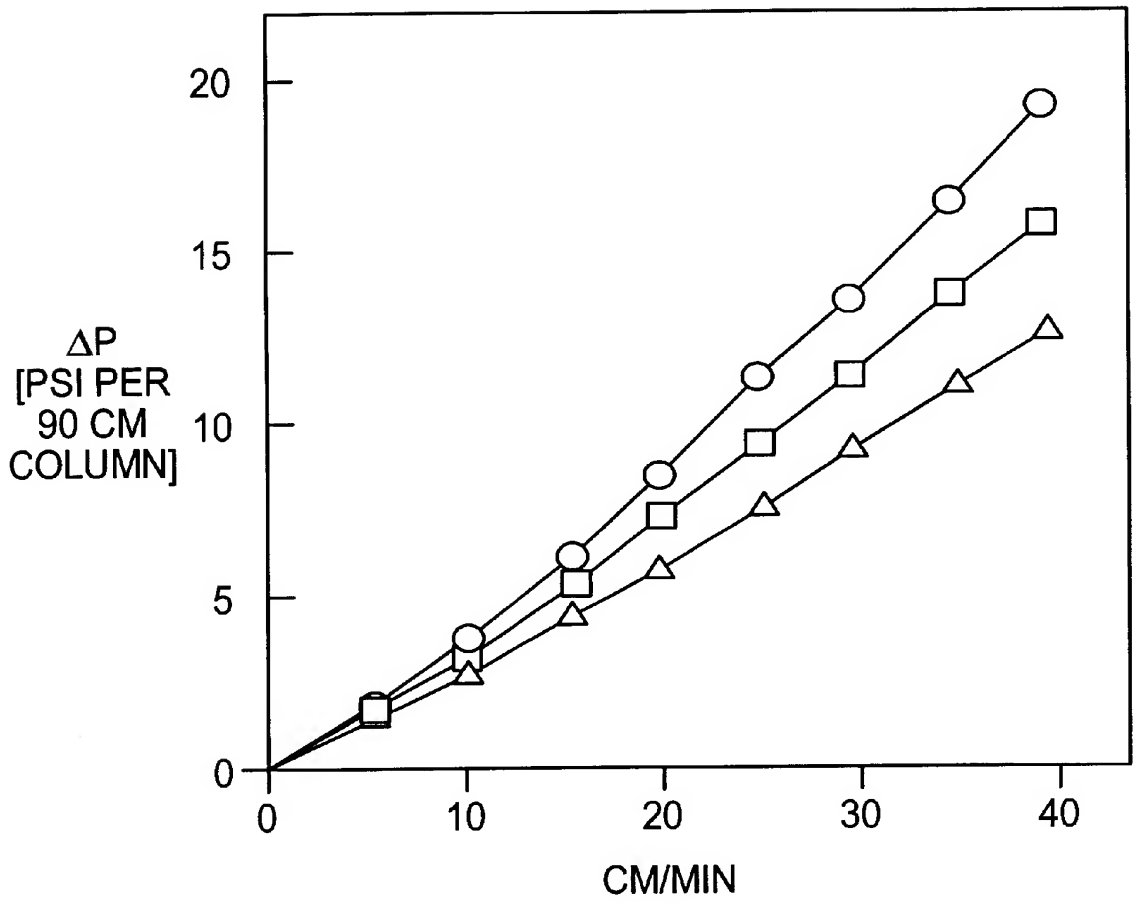

20
15
10
5
0
ΔP
[PSI PER
90 CM
COLUMN]
0
10
20
30
40
CM/MIN
2% CL-DEAE CELLULOSE BEADS
6% CL-DEAE CELLULOSE BEADS
10% CL-DEAE CELLULOSE BEADS

FIG. 6(A)

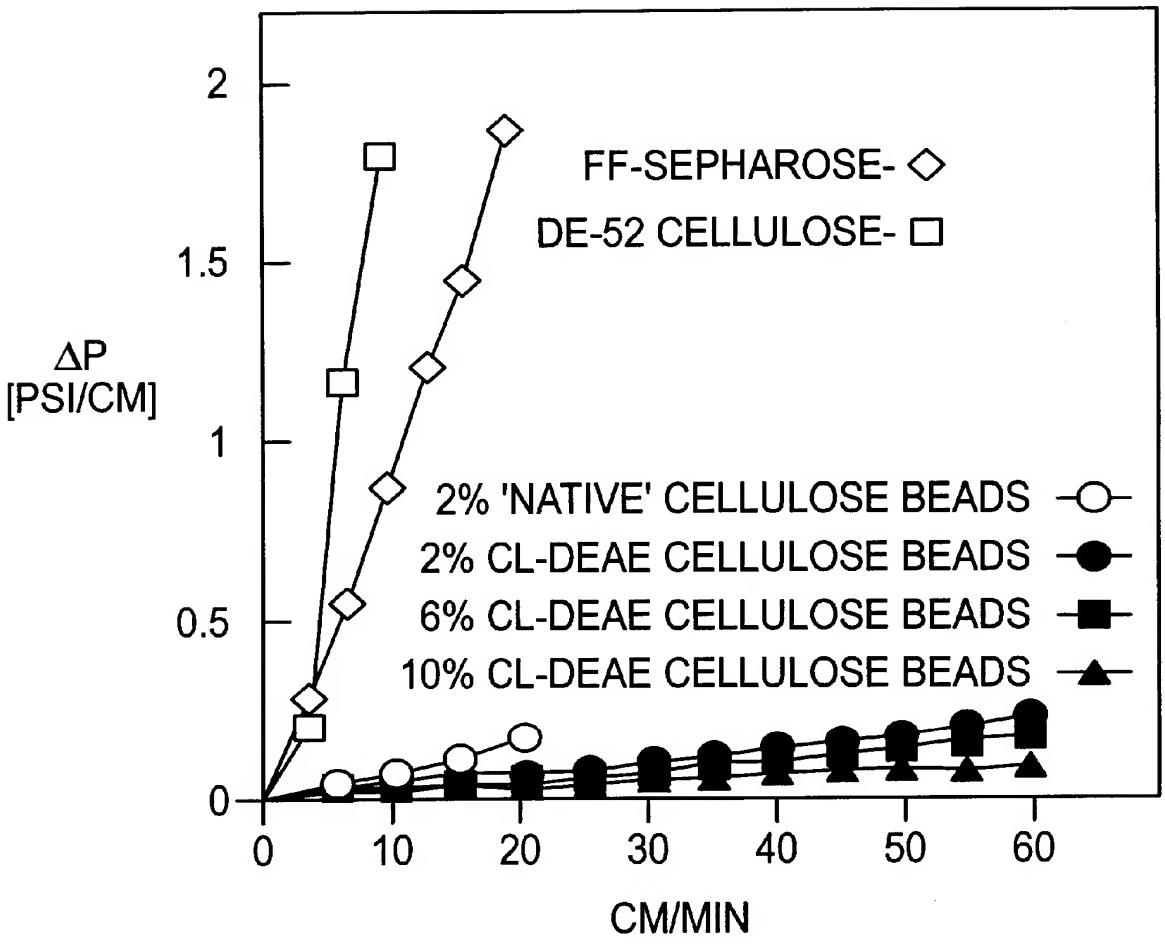

2
1.5
1
0.5
0
ΔP
[PSI/CM]
FF-SEPHAROSE-
DE-52 CELLULOSE-
2% 'NATIVE' CELLULOSE BEADS
2% CL-DEAE CELLULOSE BEADS
6% CL-DEAE CELLULOSE BEADS
10% CL-DEAE CELLULOSE BEADS
0
10
20
30
40
50
60
CM/MIN

FIG. 6(B)

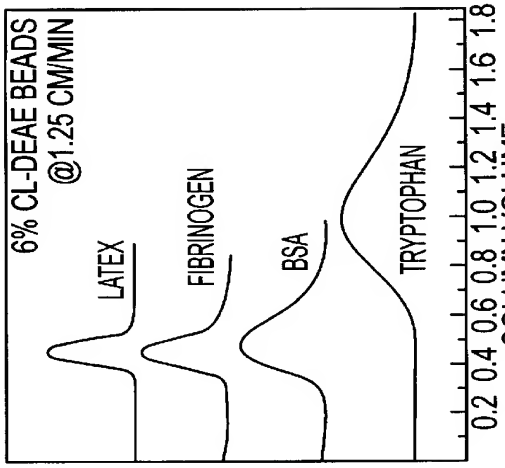


FIG. 7(A)

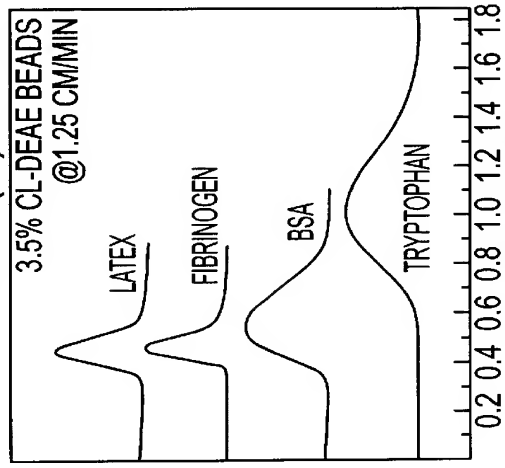


FIG. 7(C)

FIG. 7(E)

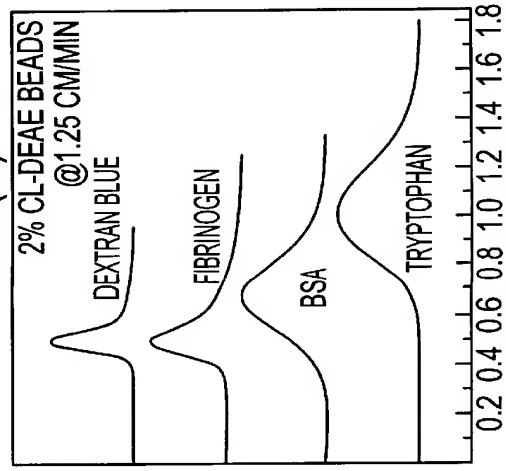


FIG. 7(B)

FIG. 7(D)

FIG. 7(F)

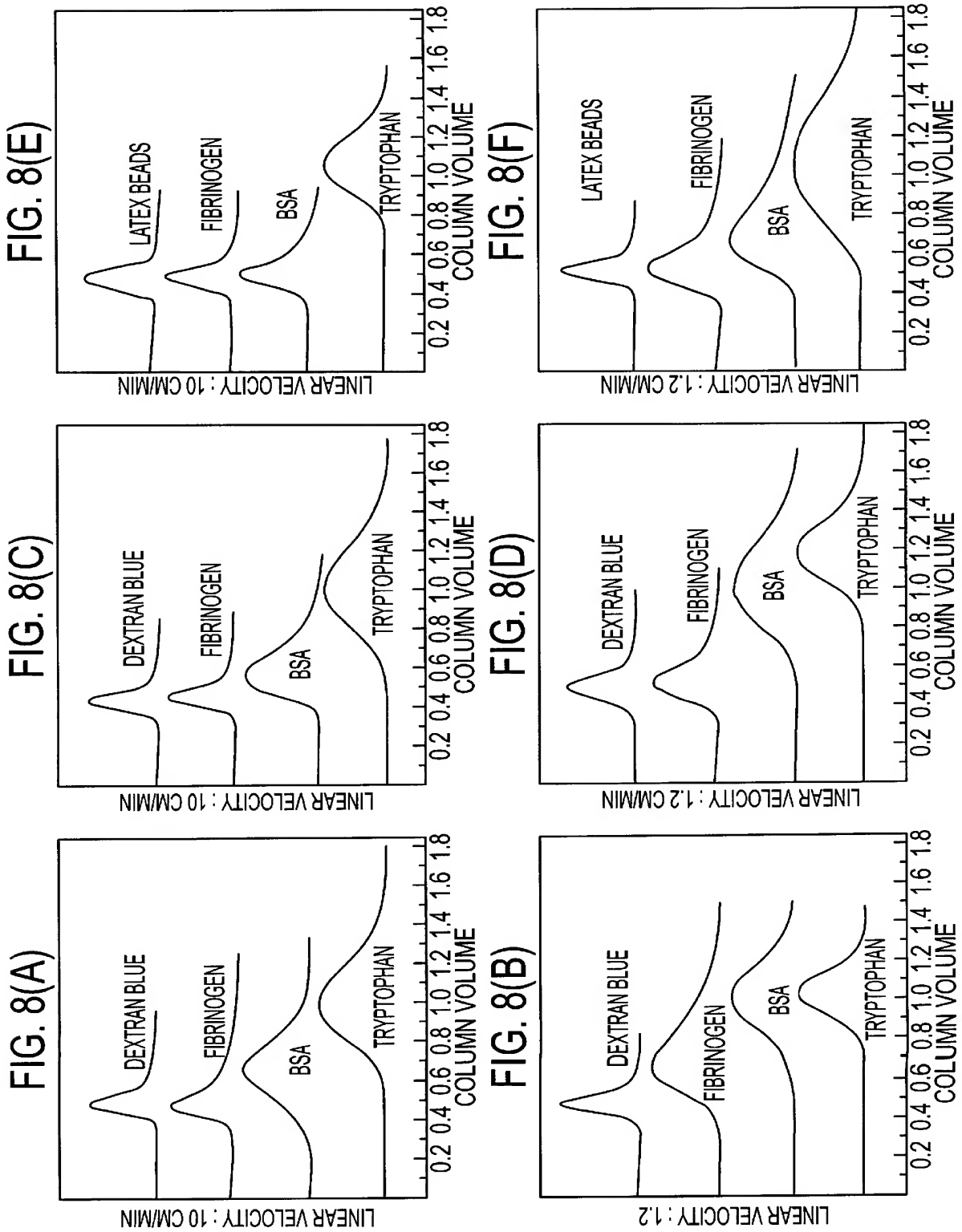


FIG. 8(A)
DEXTRAN BLUE
FIBRINOGEN
BSA
TRYPTOPHAN
LINEAR VELOCITY : 10 CM/MIN
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8
COLUMN VOLUME
FIG. 8(B)
DEXTRAN BLUE
FIBRINOGEN
BSA
TRYPTOPHAN
LINEAR VELOCITY : 1.2
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8
COLUMN VOLUME
FIG. 8(C)
DEXTRAN BLUE
FIBRINOGEN
BSA
TRYPTOPHAN
LINEAR VELOCITY : 10 CM/MIN
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8
COLUMN VOLUME
FIG. 8(D)
DEXTRAN BLUE
FIBRINOGEN
BSA
TRYPTOPHAN
LINEAR VELOCITY : 1.2 CM/MIN
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8
COLUMN VOLUME
FIG. 8(E)
LATEX BEADS
FIBRINOGEN
BSA
TRYPTOPHAN
LINEAR VELOCITY : 10 CM/MIN
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8
COLUMN VOLUME
FIG. 8(F)
LATEX BEADS
FIBRINOGEN
BSA
TRYPTOPHAN
LINEAR VELOCITY : 1.2 CM/MIN
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8
COLUMN VOLUME

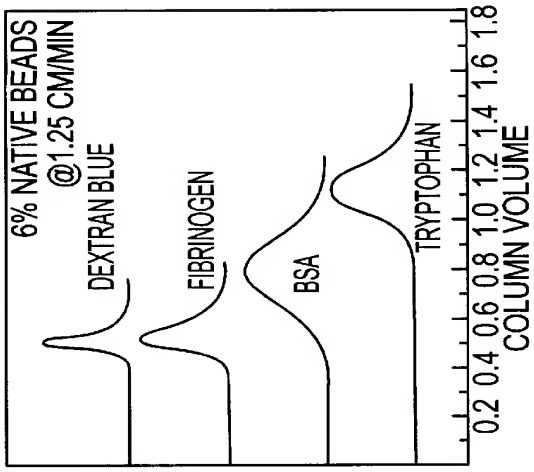


FIG. 9(E)

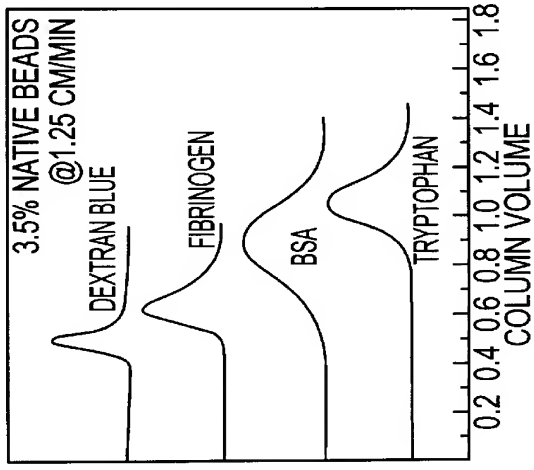


FIG. 9(C)

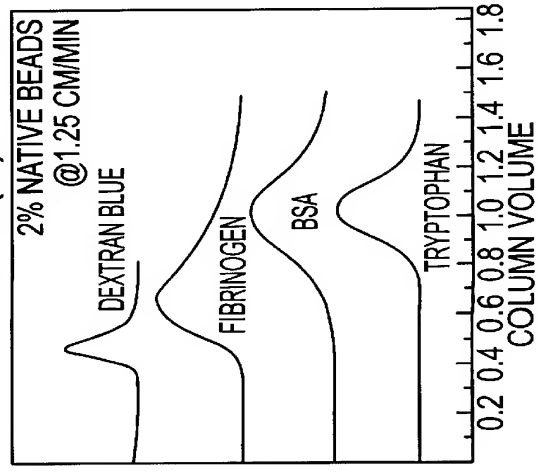


FIG. 9(A)

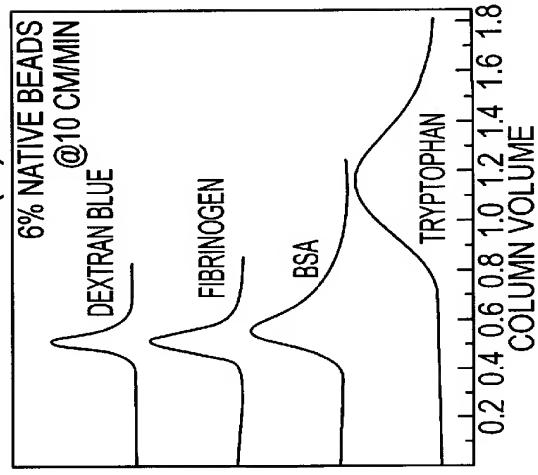


FIG. 9(F)

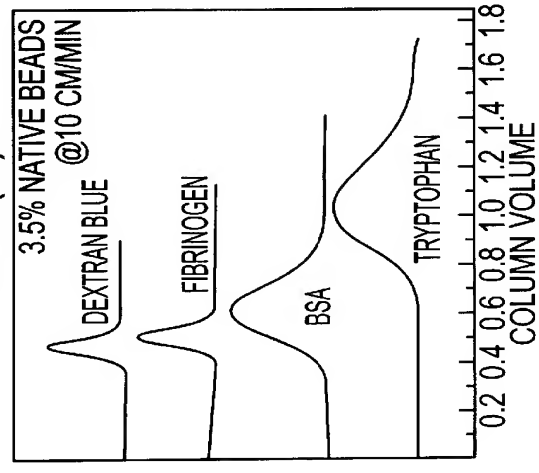


FIG. 9(D)

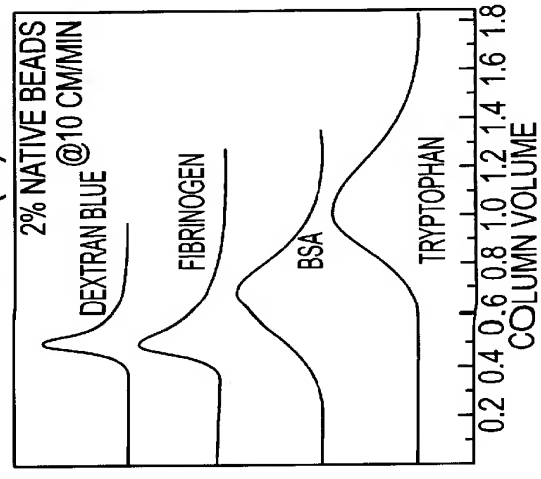


FIG. 9(B)

FIG. 10 (A)

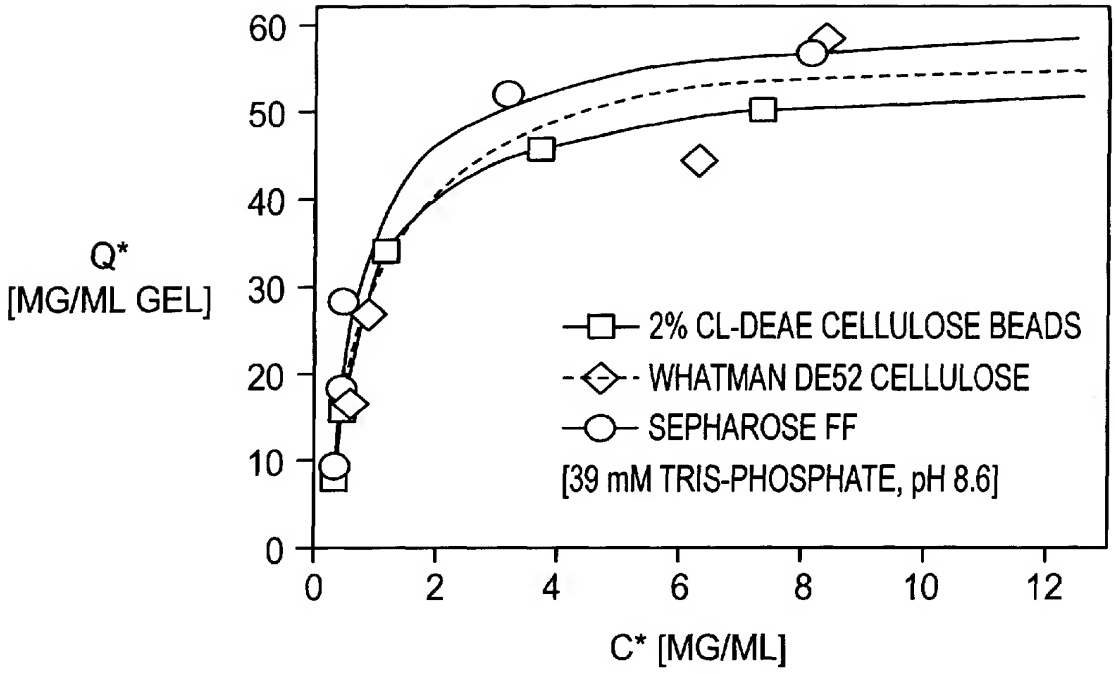


FIG. 10 (B)

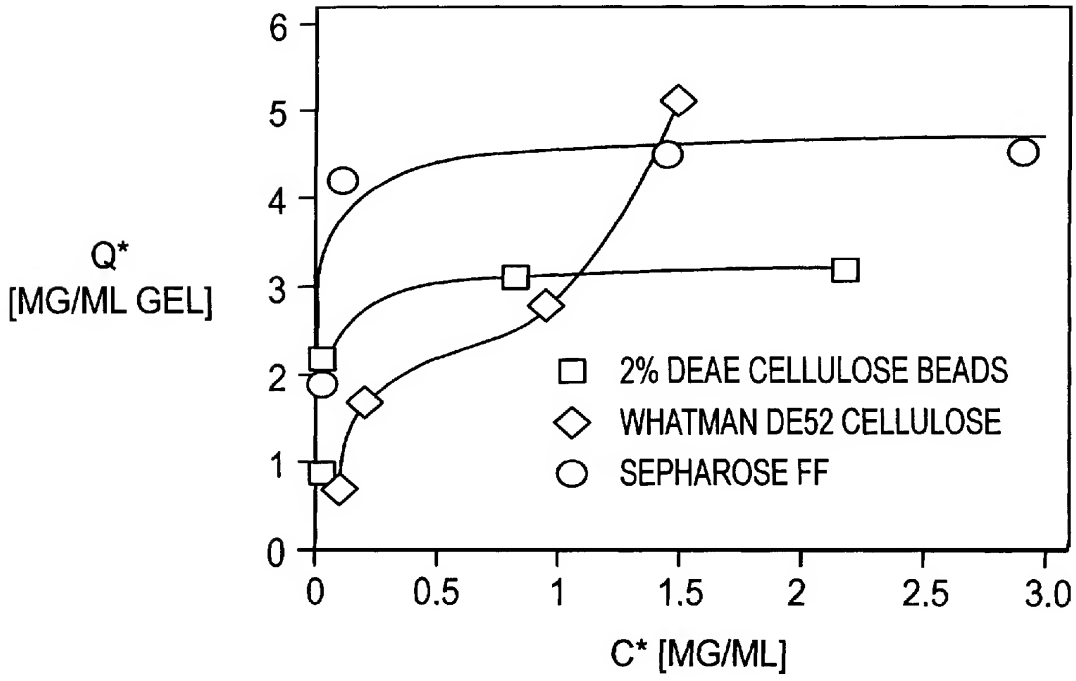


FIG. 11

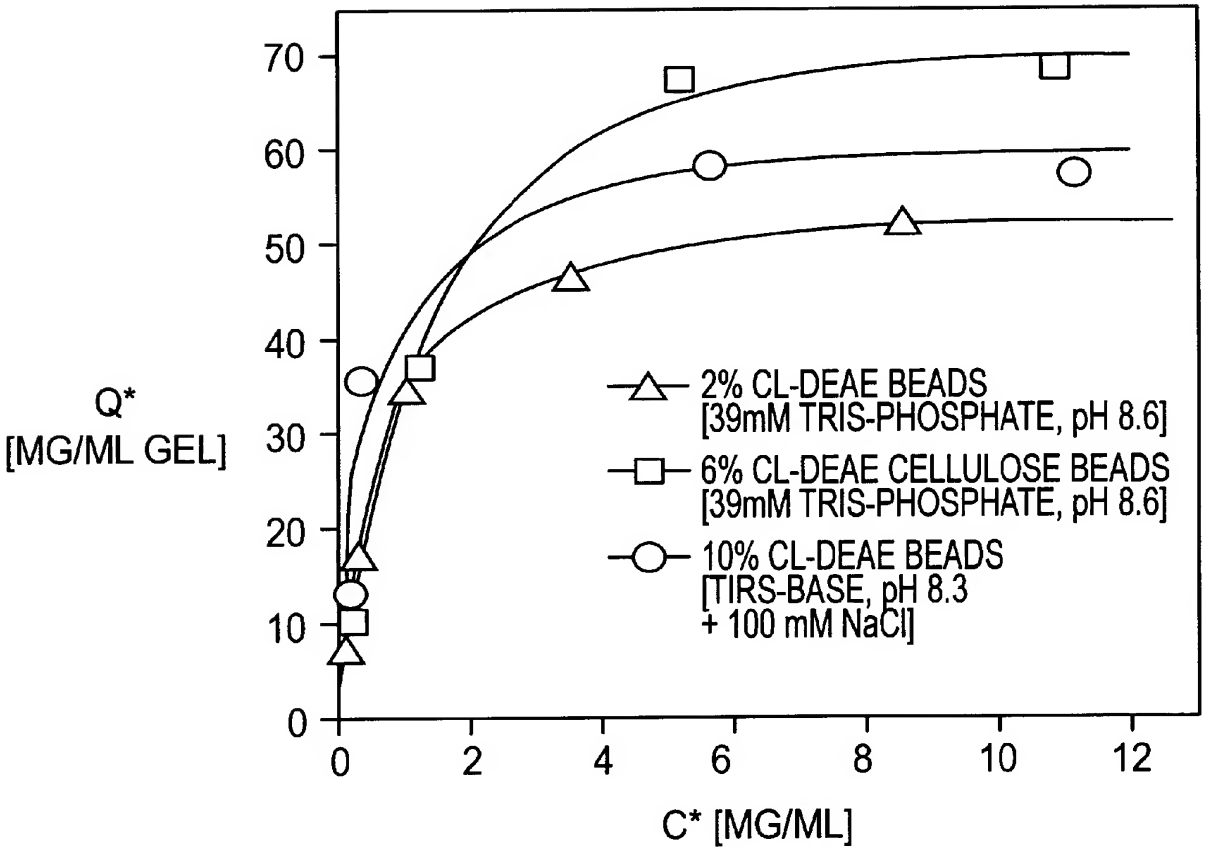

Q*
[MG/ML GEL]
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
C* [MG/ML]
2% CL-DEAE BEADS
[39mM TRIS-PHOSPHATE, pH 8.6]
6% CL-DEAE CELLULOSE BEADS
[39mM TRIS-PHOSPHATE, pH 8.6]
10% CL-DEAE BEADS
[TIRS-BASE, pH 8.3
+ 100 mM NaCl]

FIG. 12 (A)

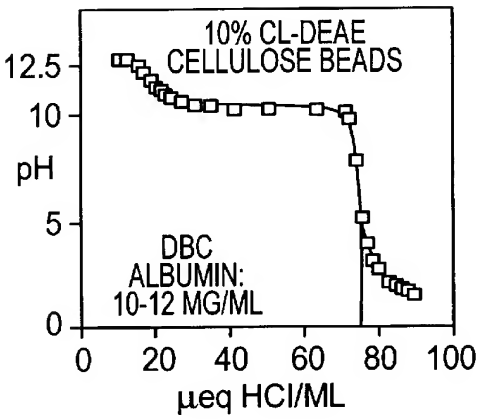


FIG. 12 (B)

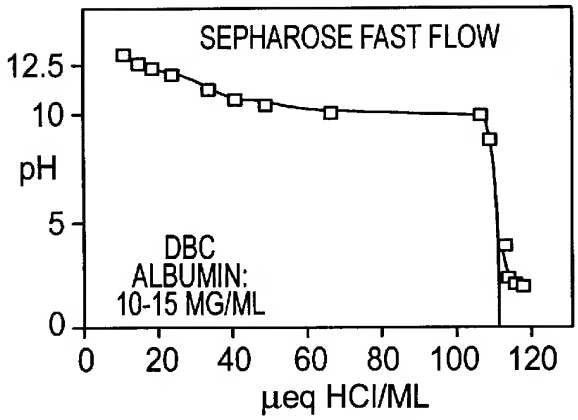


FIG. 12 (C)

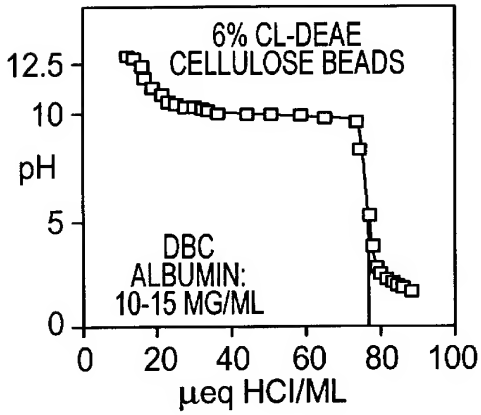


FIG. 12 (D)

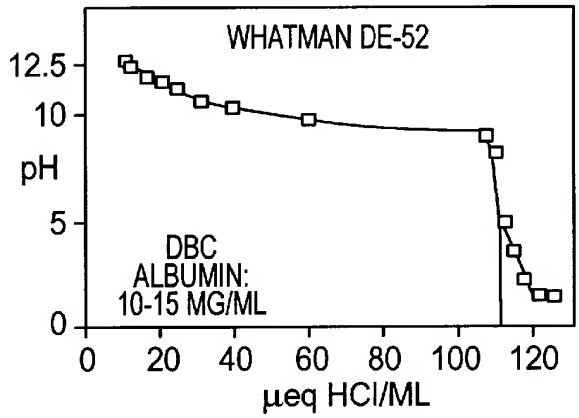


FIG. 12 (E)

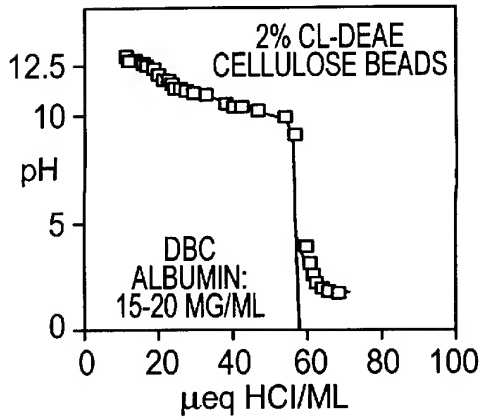


FIG. 12 (F)

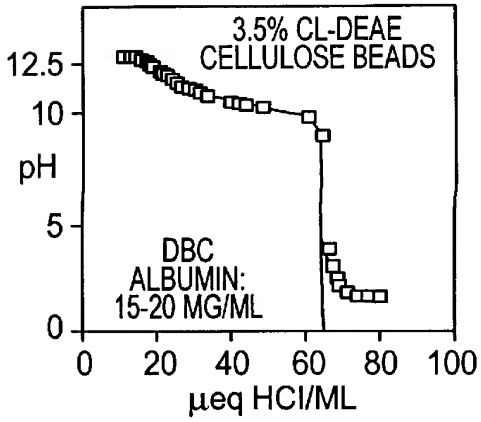


FIG. 13 (A)

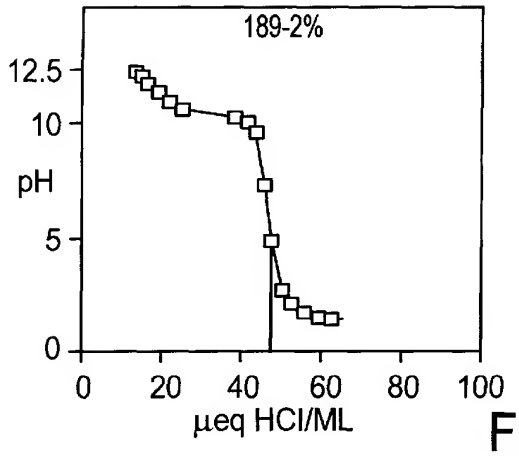


FIG. 13 (B)

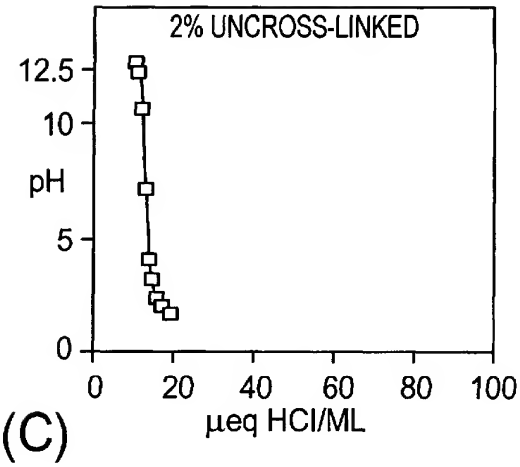


FIG. 13 (C)

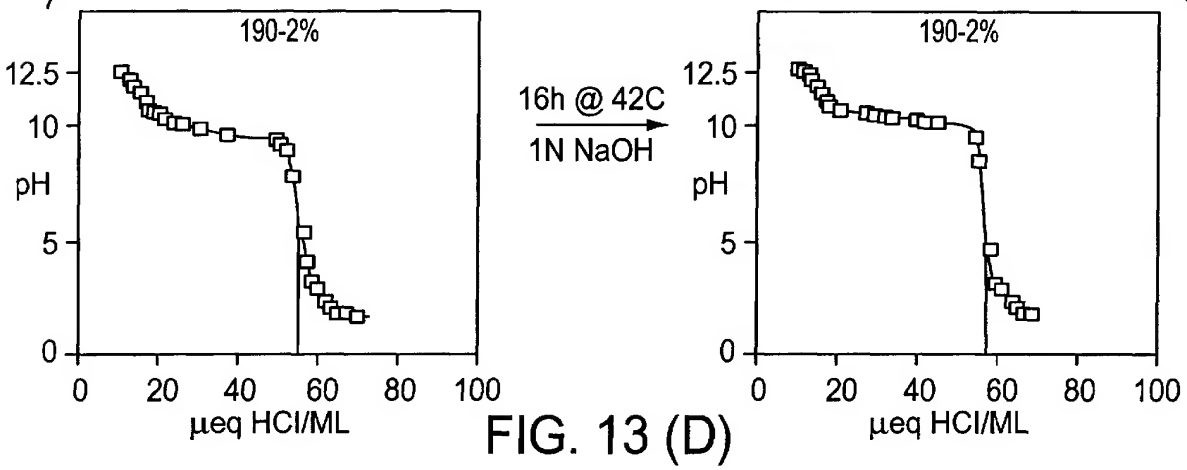


FIG. 13 (D)

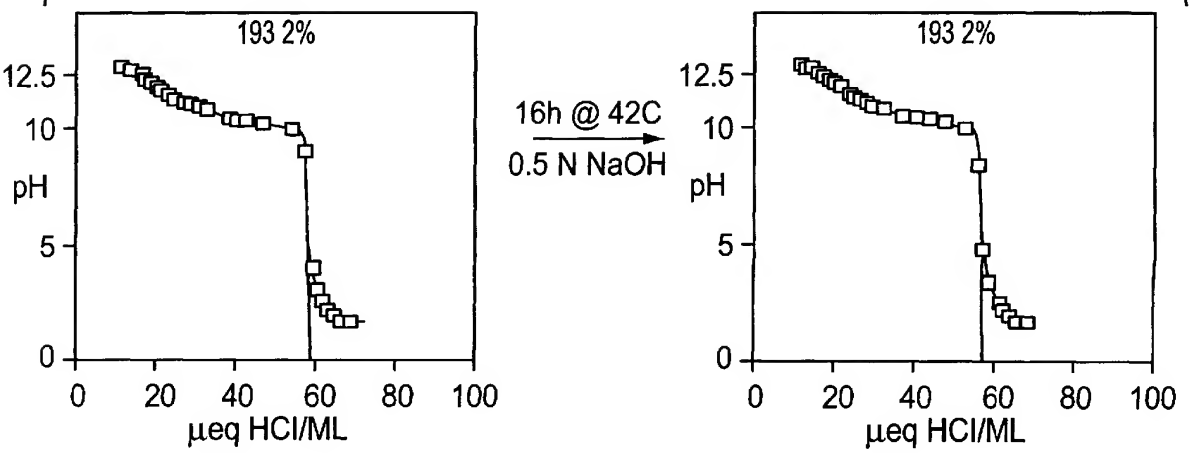


FIG. 14 (A)

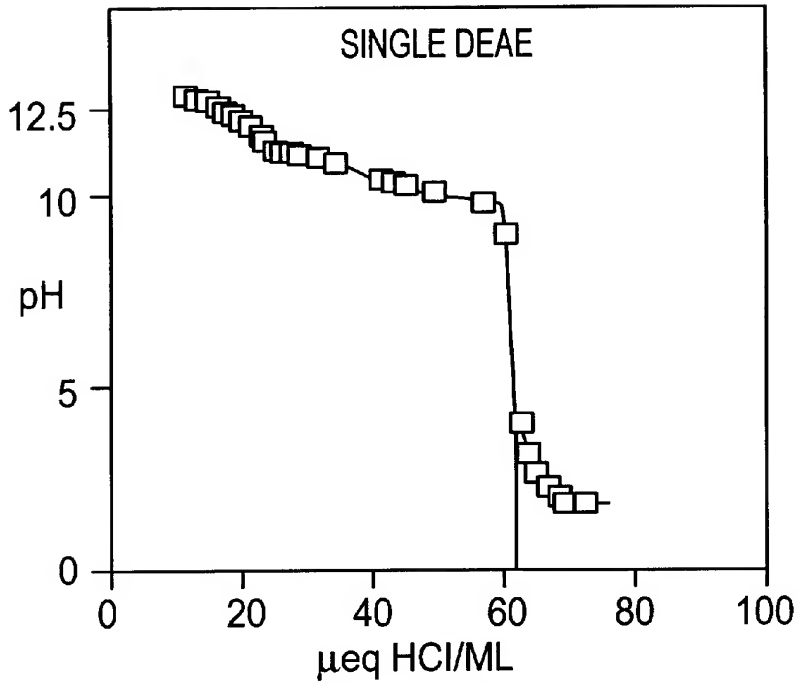


FIG. 14 (B)

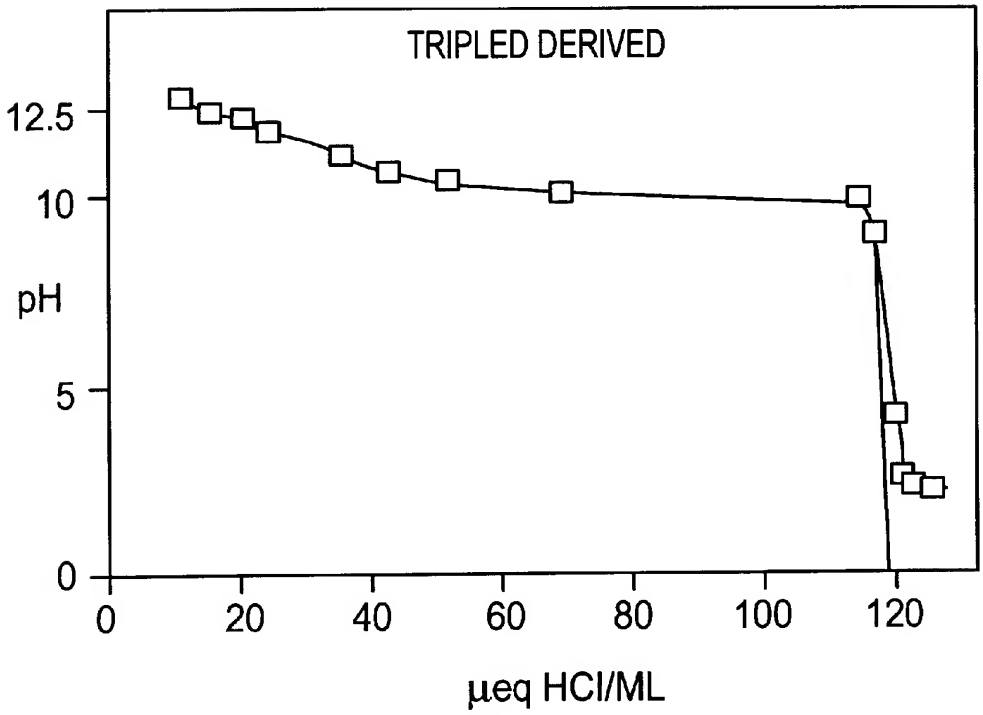


FIG. 15(A)

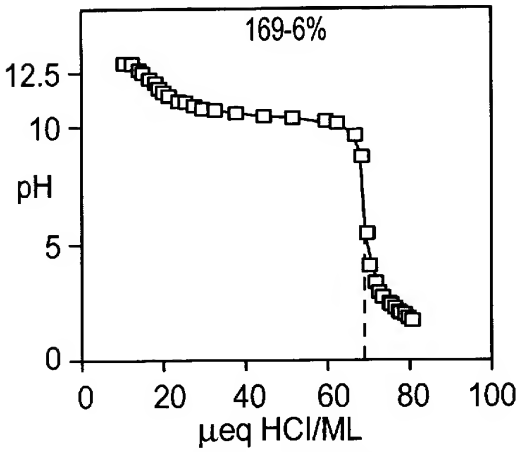


FIG. 15(B)

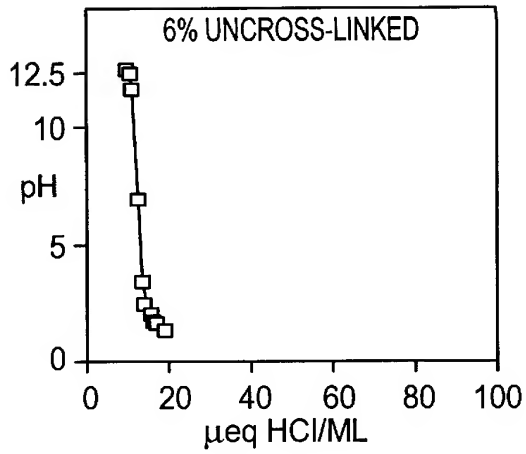


FIG. 15(C)

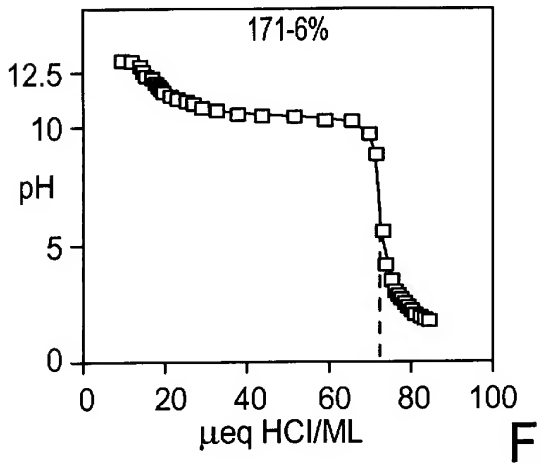


FIG. 15(D)

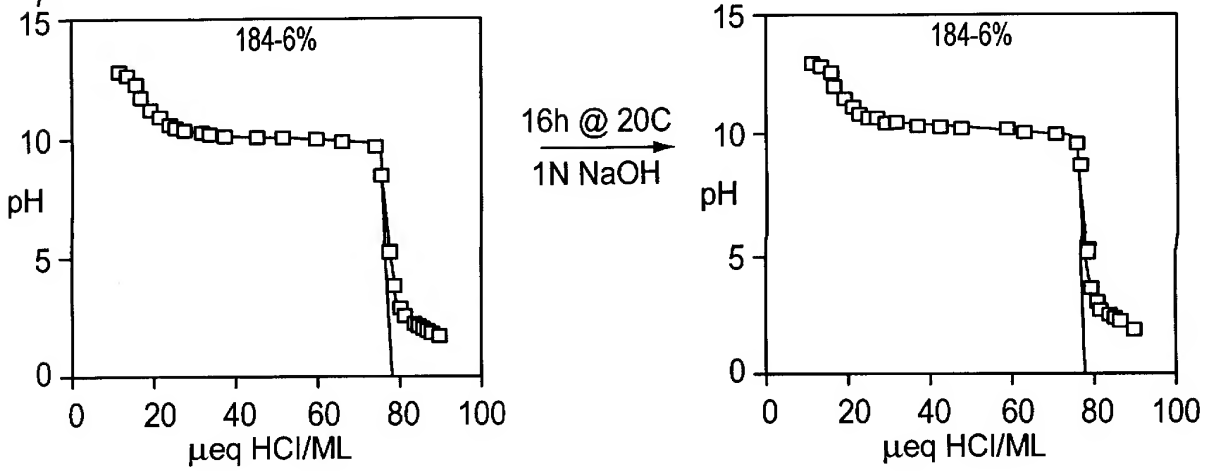


FIG. 16(A)

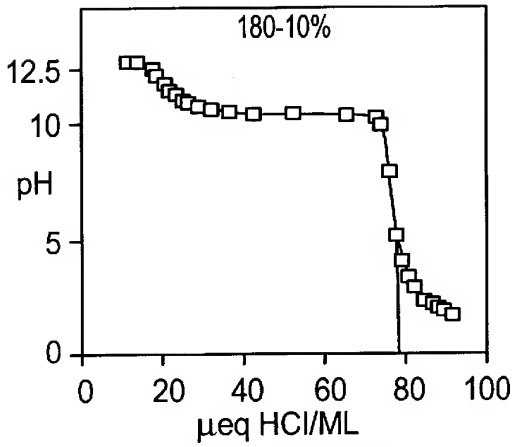


FIG. 16(B)

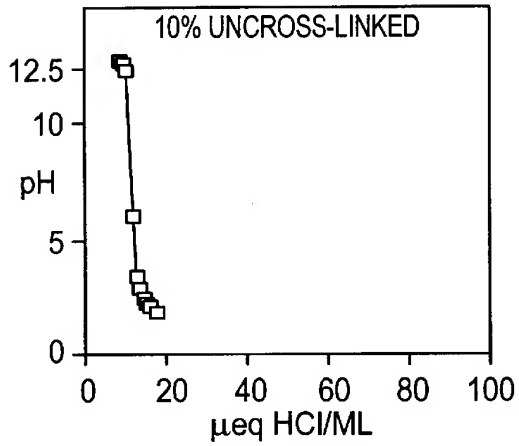


FIG. 16(C)

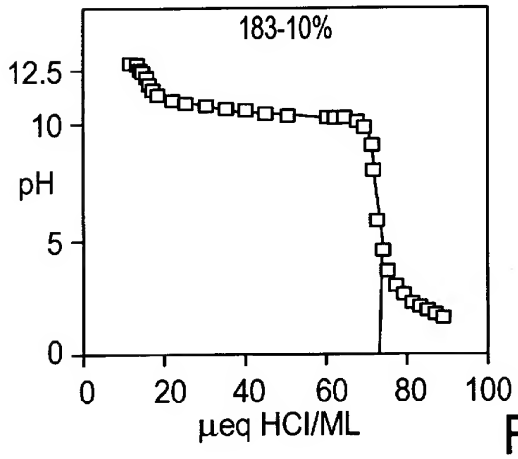


FIG. 16(D)

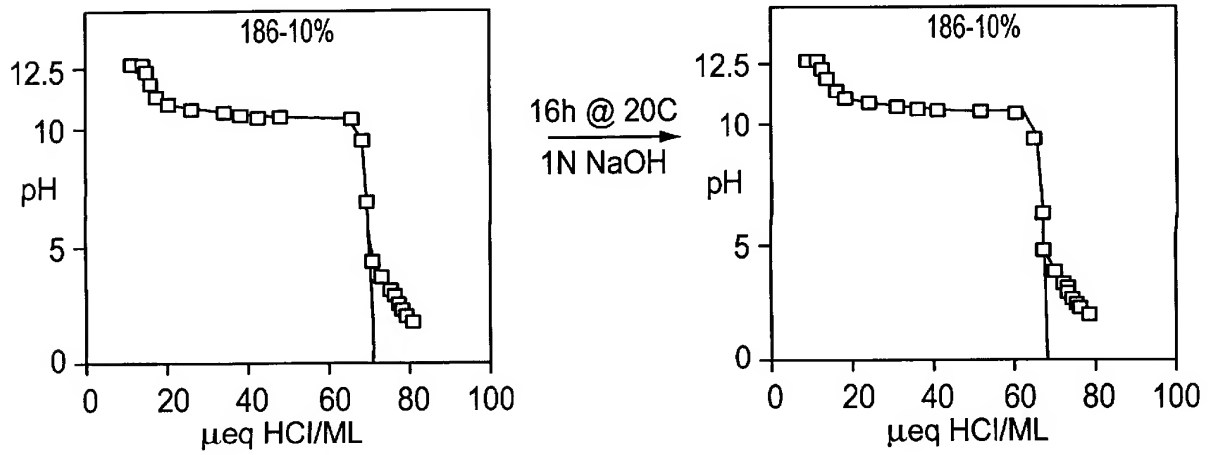


FIG. 17(A)

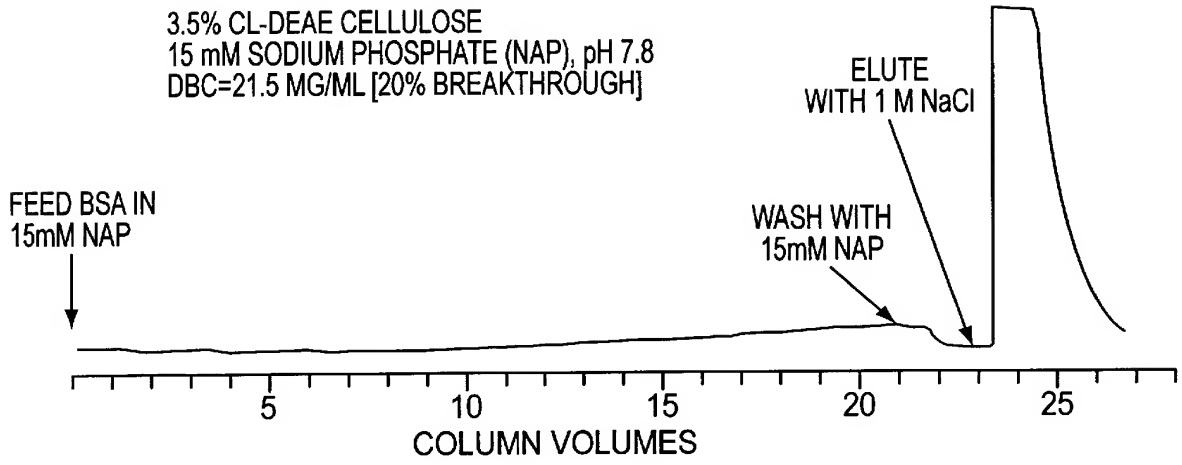


FIG. 17(B)

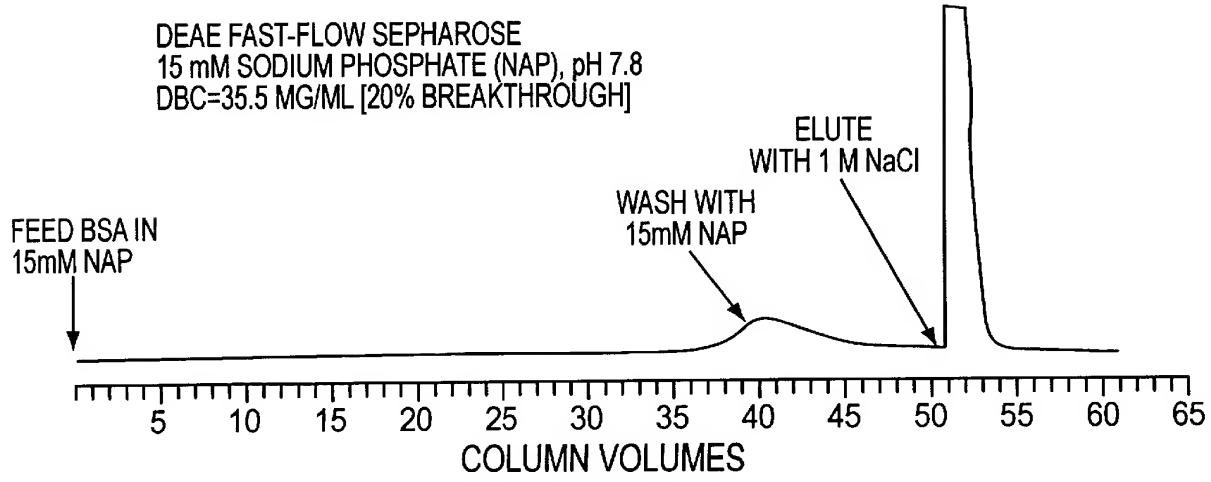


FIG. 18(A)

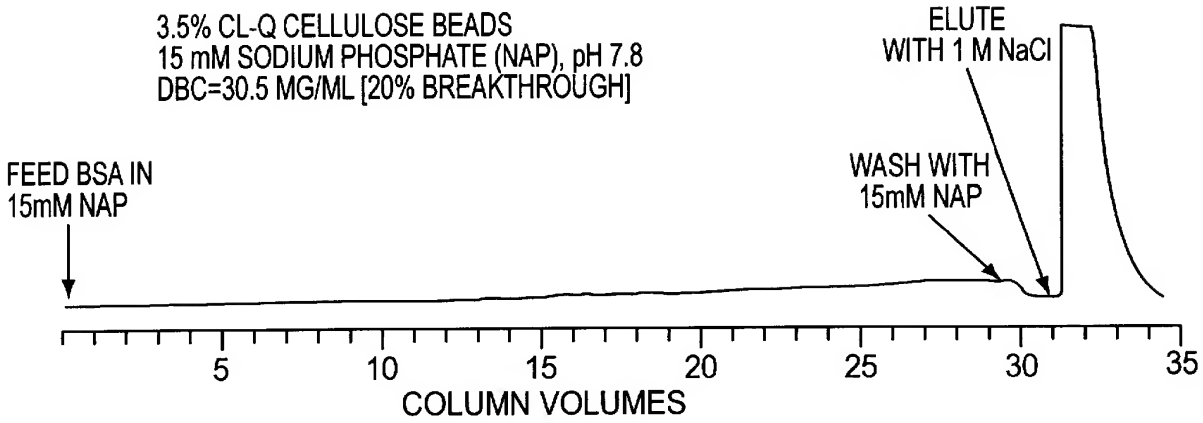


FIG. 18(B)

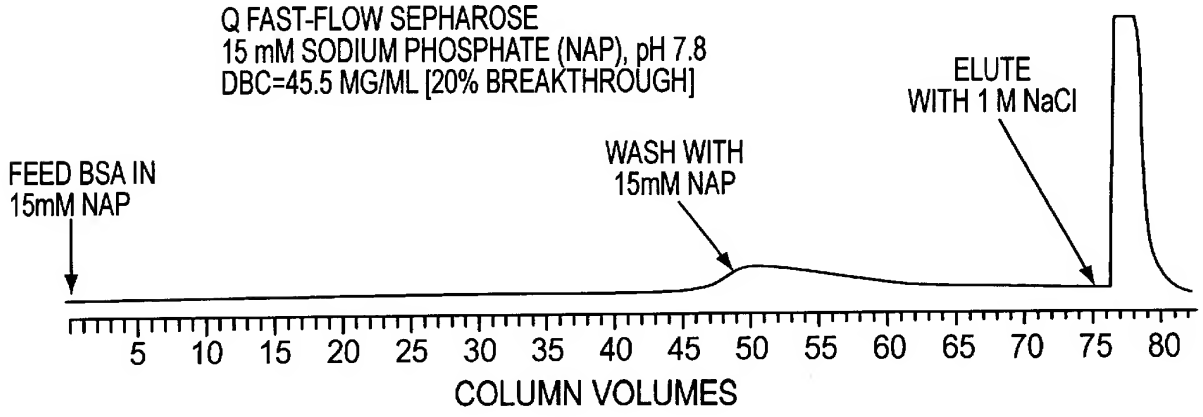
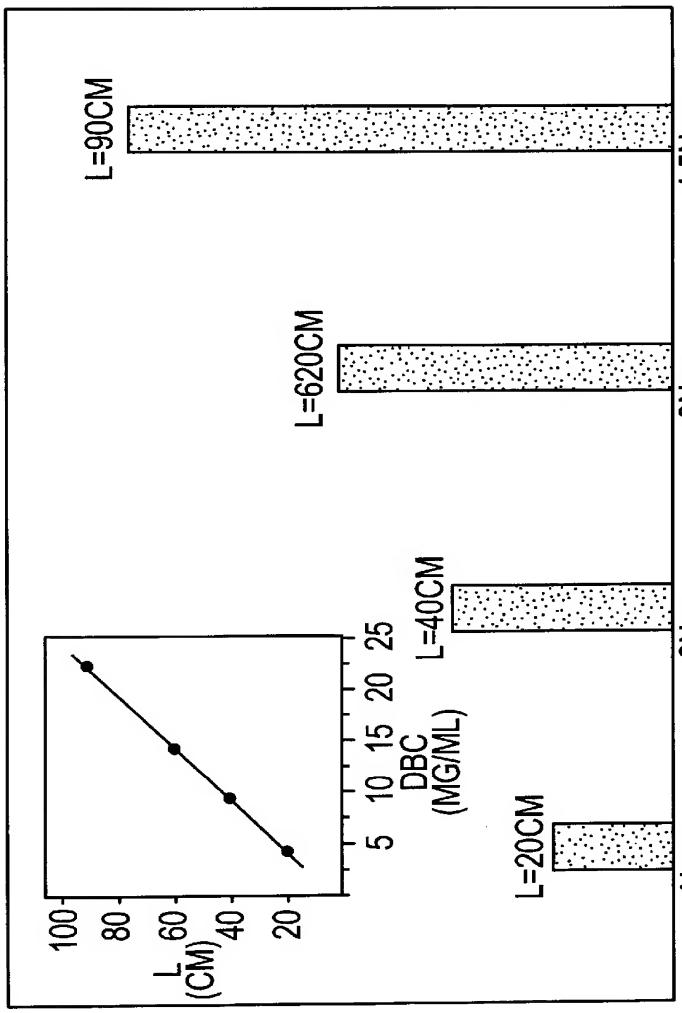


FIG. 19



Mg ALBUMIN PER ML PACKING	4.3 MG/ML	9.6 MG/ML	14.7 MG/ML	23 MG/ML
TOTAL ALBUMIN CAPTURED	172 MG TOTAL	768 MG TOTAL	1764 MG TOTAL	4140 MG TOTAL
	86% YIELD	88% YIELD	92% YIELD	95% YIELD
	[ΔP=0.8 PSI]	[ΔP=1.1 PSI]	[ΔP=1.6 PSI]	[ΔP=2.4 PSI]

FIG. 20(A)

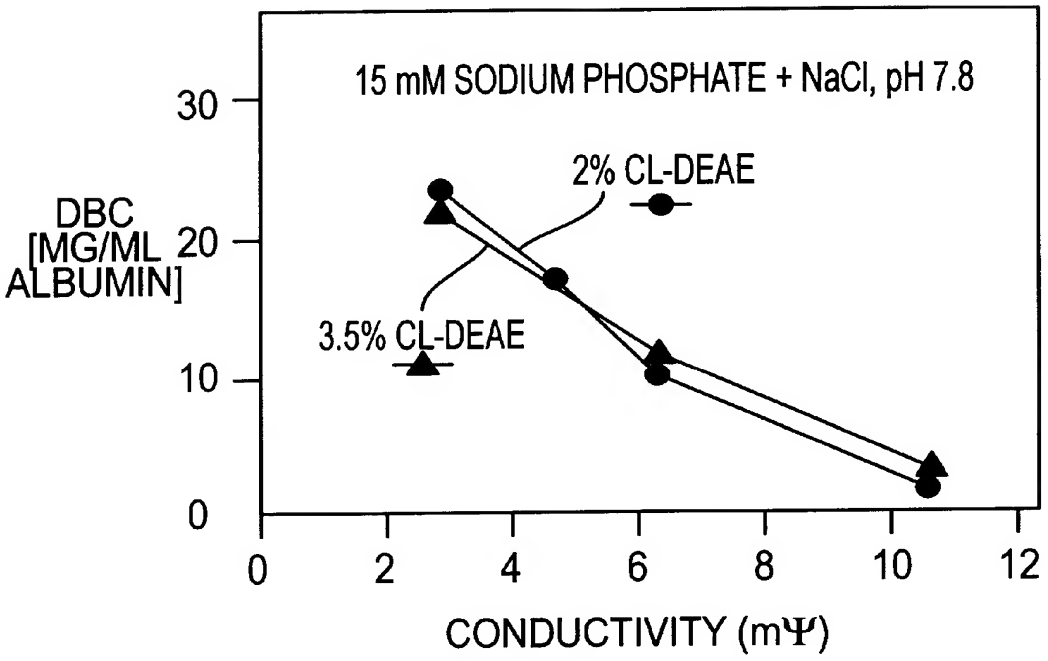


FIG. 20(B)

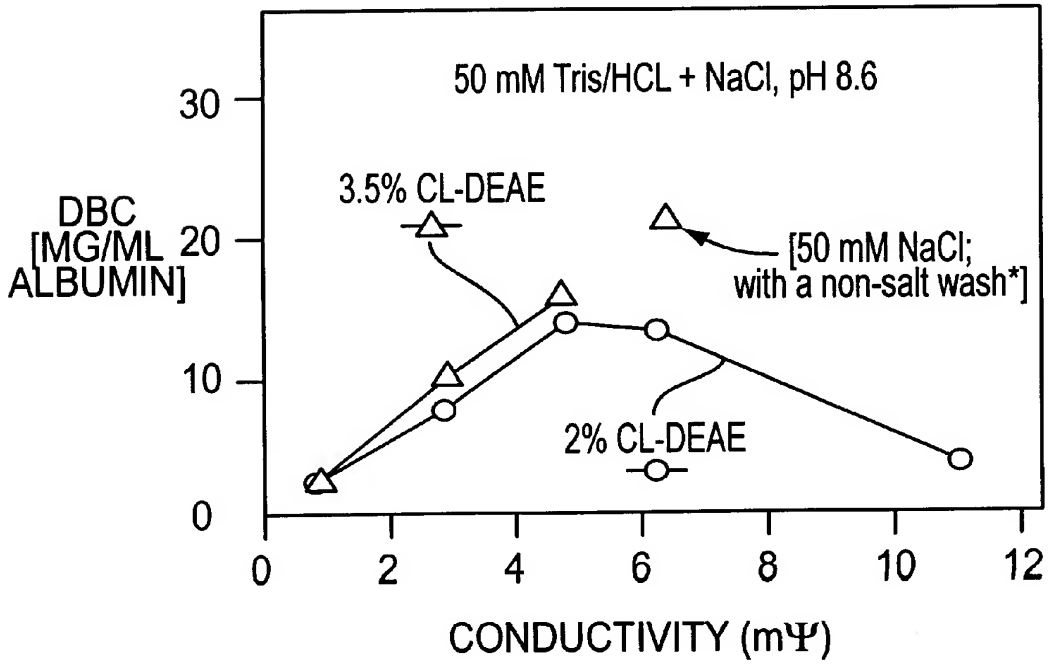


FIG. 21

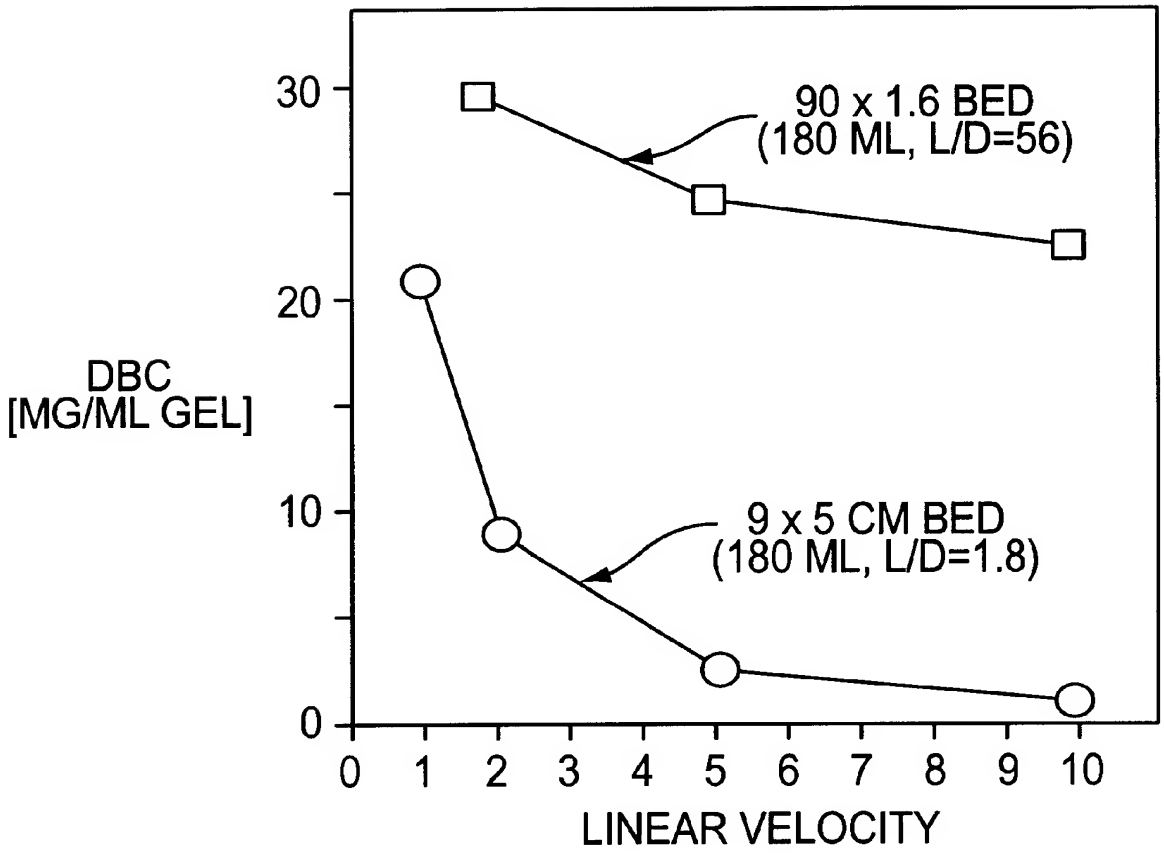

30
20
10
0
DBC
[MG/ML GEL]
90 x 1.6 BED
(180 ML, L/D=56)
9 x 5 CM BED
(180 ML, L/D=1.8)
0 1 2 3 4 5 6 7 8 9 10
LINEAR VELOCITY

FIG. 22

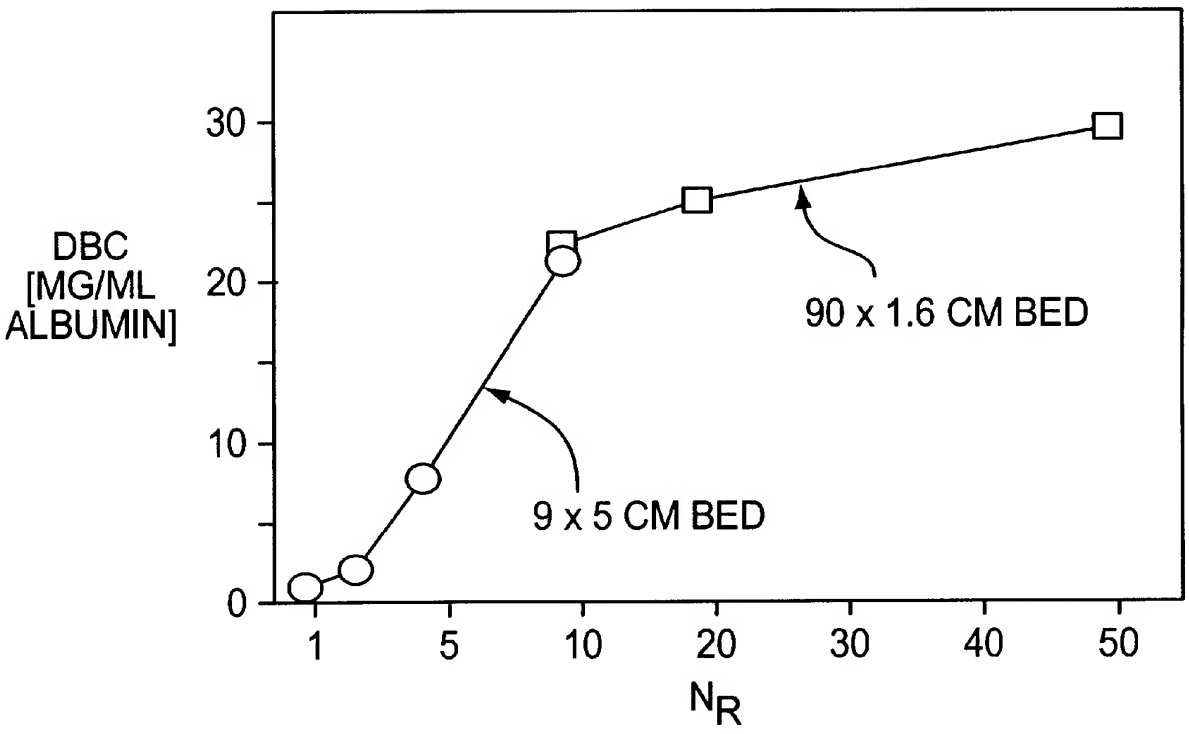

DBC
[MG/ML
ALBUMIN]
30
20
10
0
1
5
10
20
30
40
50
N_R
90 x 1.6 CM BED
9 x 5 CM BED

FIG. 23

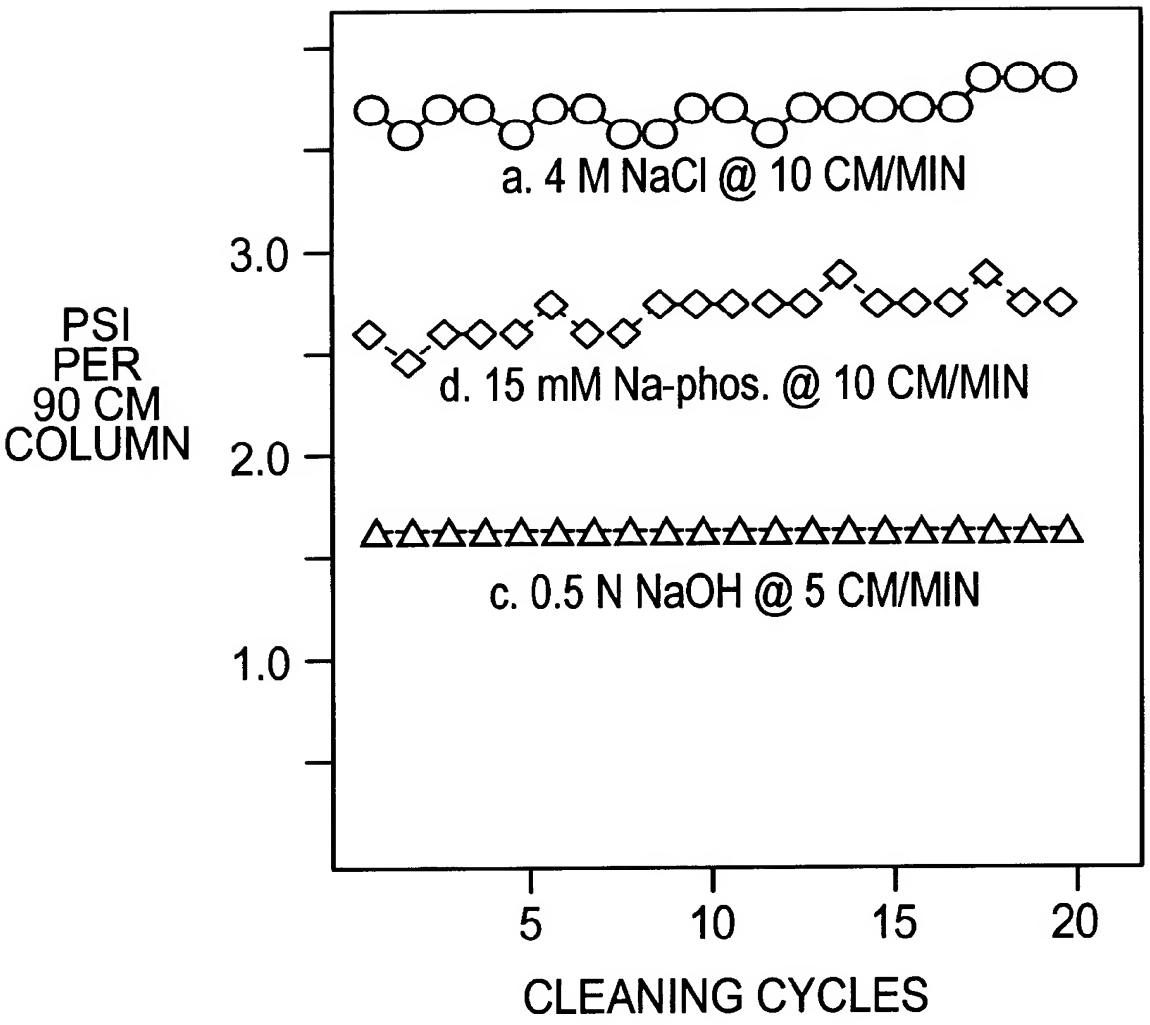

PSI
PER
90 CM
COLUMN
3.0
2.0
1.0
a. 4 M NaCl @ 10 CM/MIN
d. 15 mM Na-phos. @ 10 CM/MIN
c. 0.5 N NaOH @ 5 CM/MIN
5
10
15
20
CLEANING CYCLES

FIG. 24

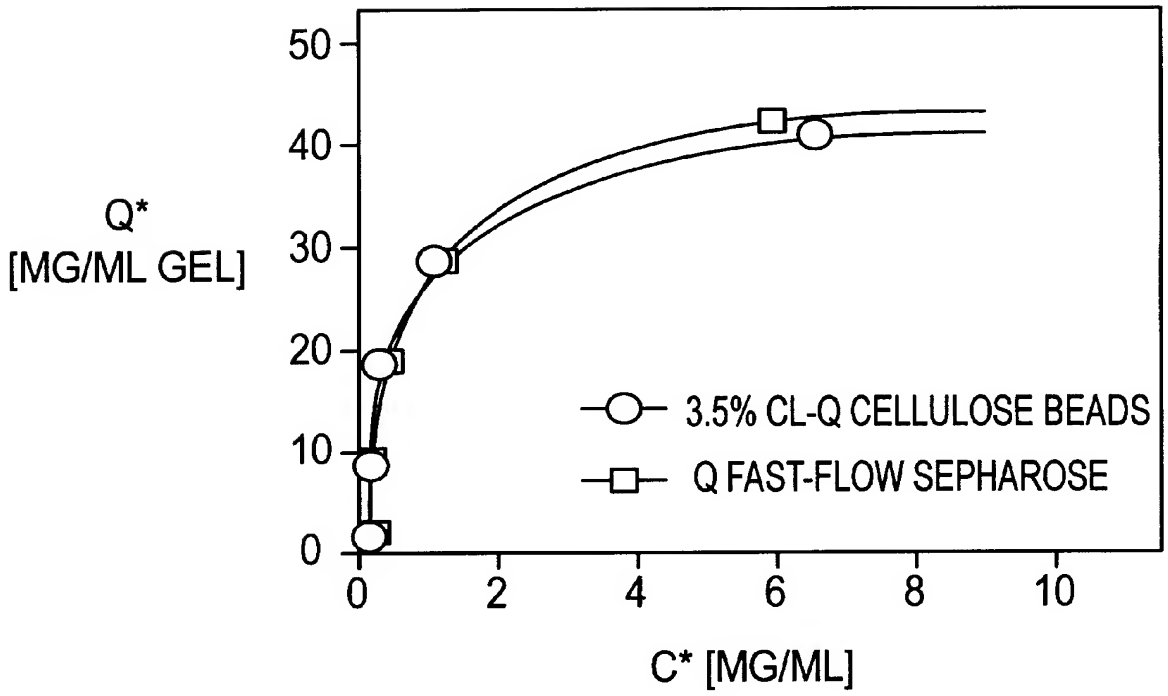

Q*
[MG/ML GEL]
50
40
30
20
10
0
0
2
4
6
8
10
C* [MG/ML]
3.5% CL-Q CELLULOSE BEADS
Q FAST-FLOW SEPHAROSE

FIG. 25(A)

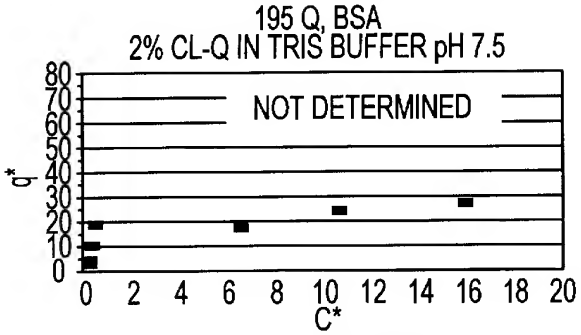


FIG. 25(B)

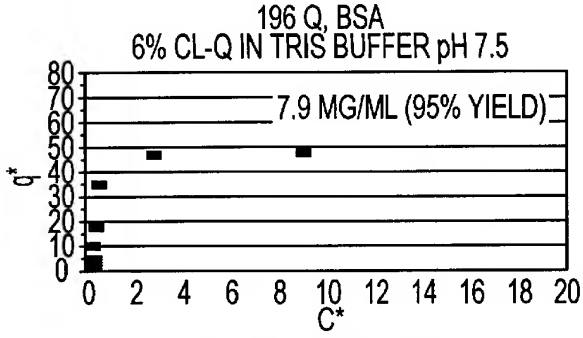


FIG. 25(C)

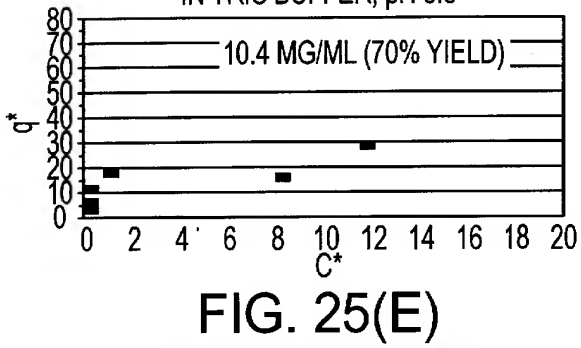


FIG. 25(D)

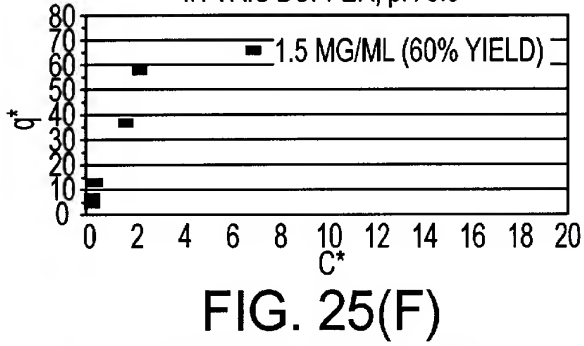


FIG. 25(E)

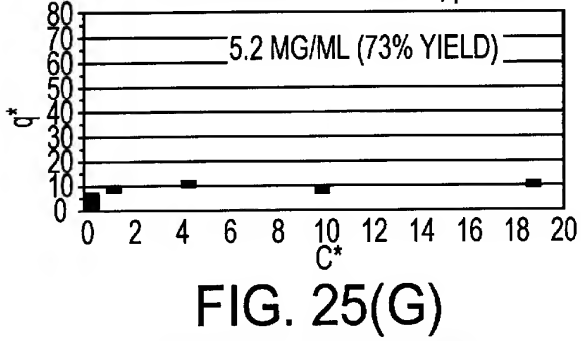


FIG. 25(F)

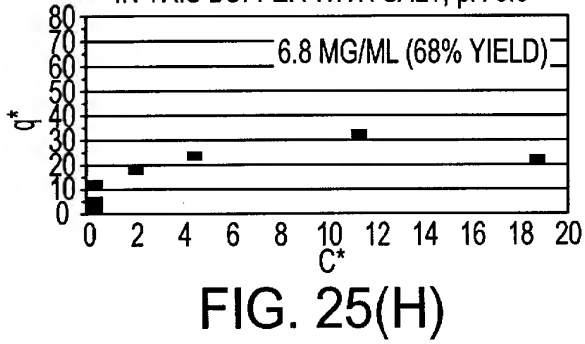


FIG. 25(G)

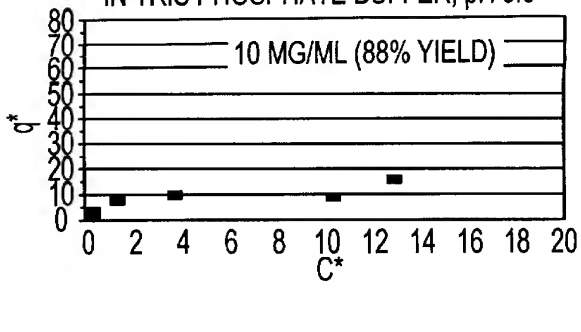


FIG. 25(H)

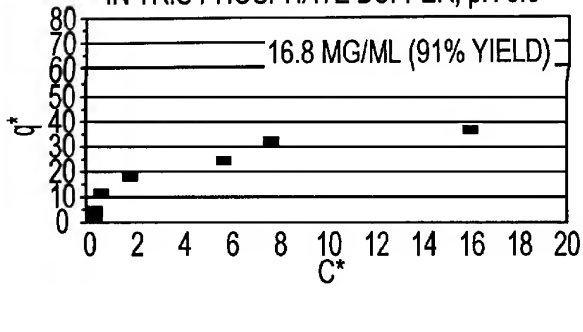


FIG. 26(A)

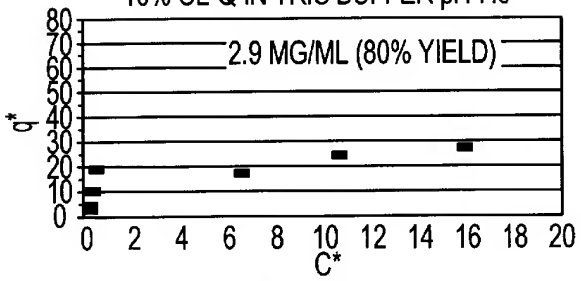


FIG. 26(B)

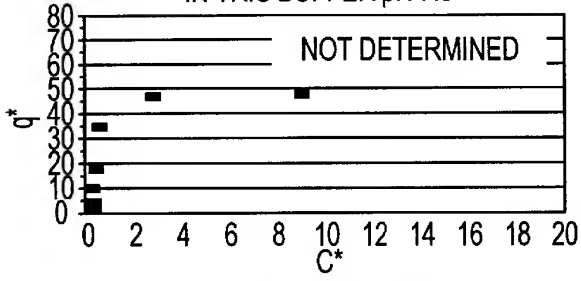


FIG. 26(C)

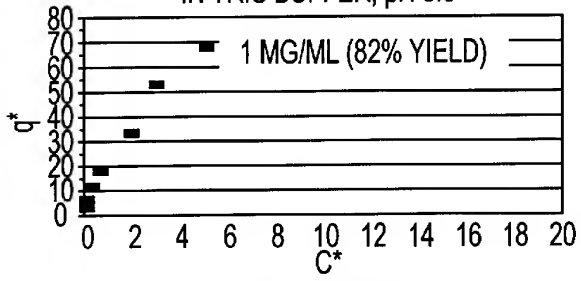


FIG. 26(D)

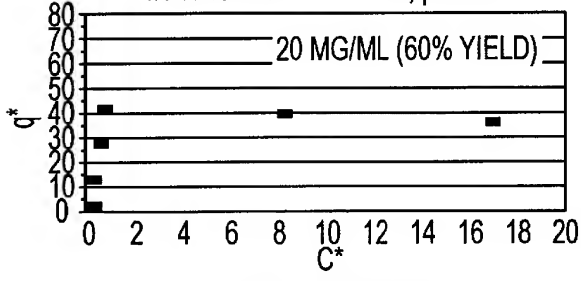


FIG. 26(E)

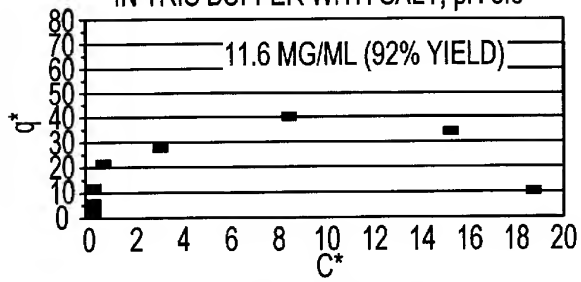


FIG. 26(F)

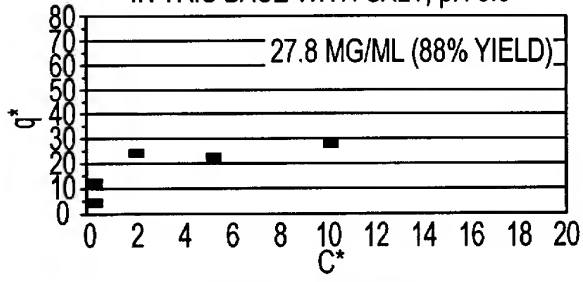


FIG. 26(G)

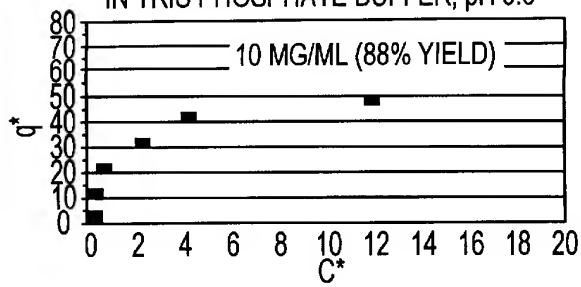
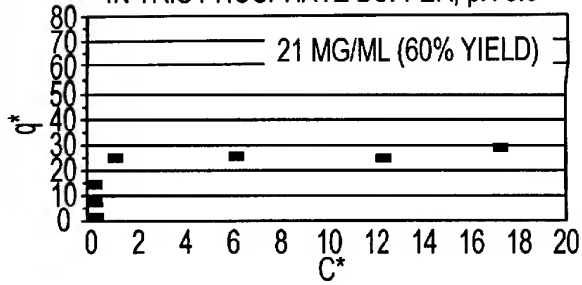


FIG. 26(H)



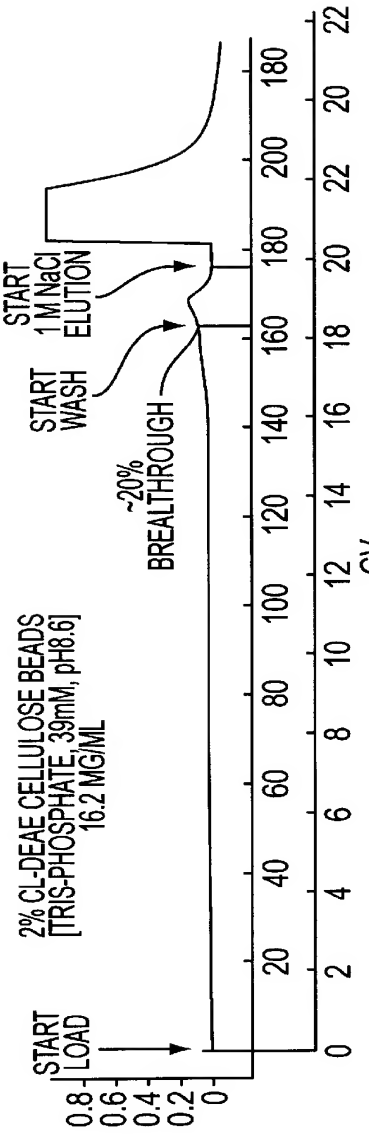


FIG. 27(A)

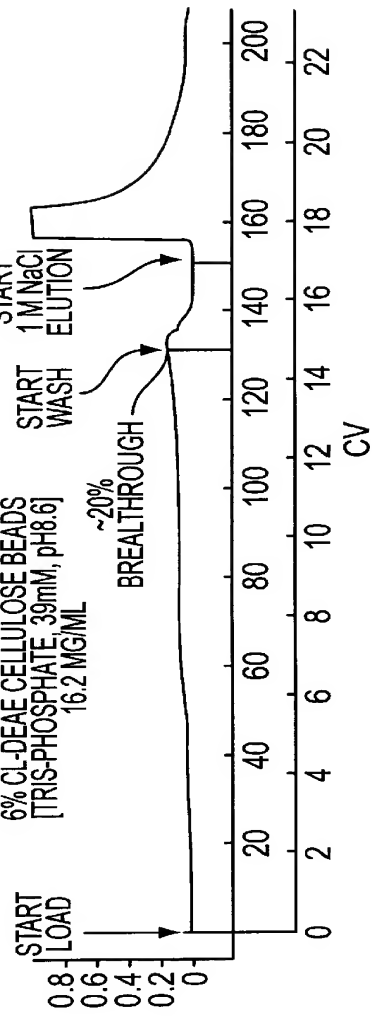


FIG. 27(B)

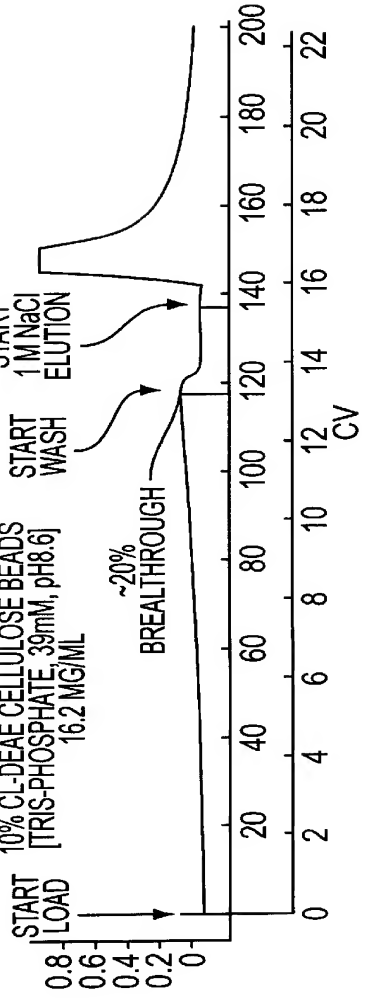


FIG. 27(C)

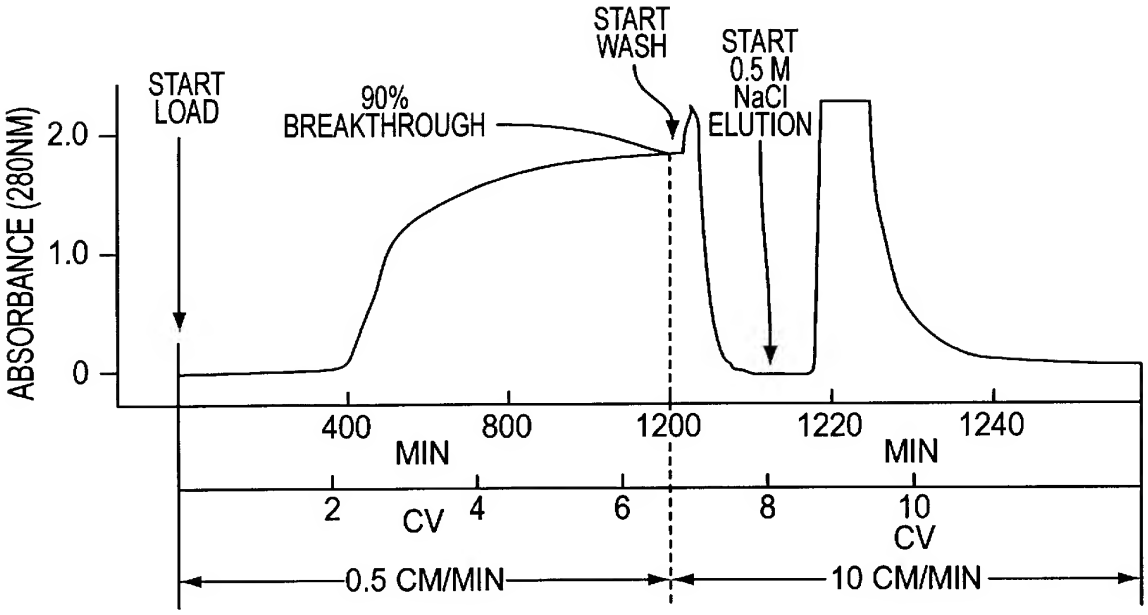


FIG. 28(A)

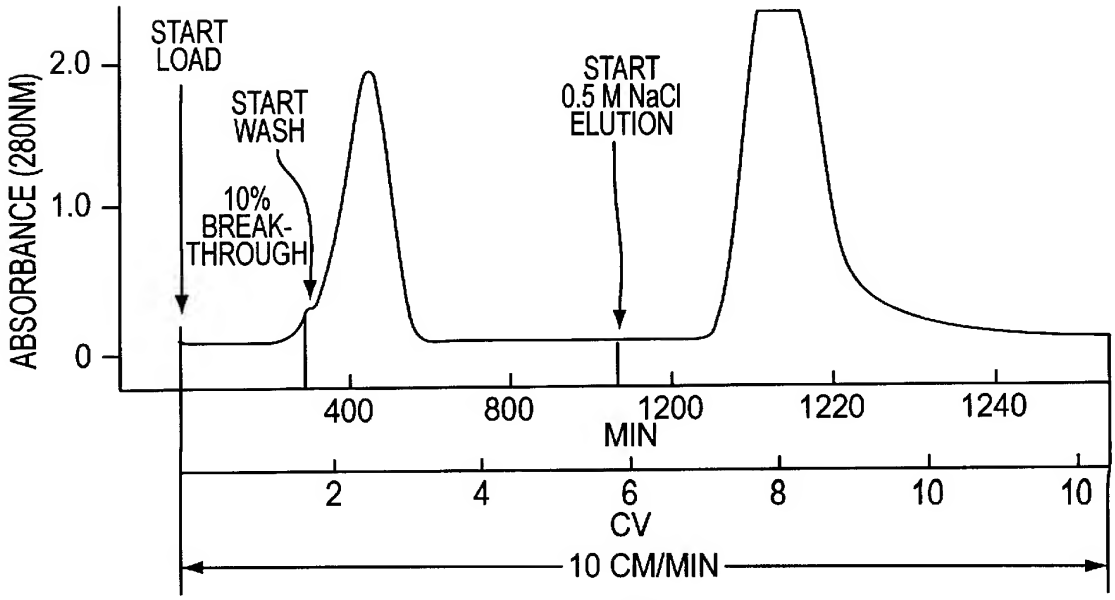


FIG. 28(B)

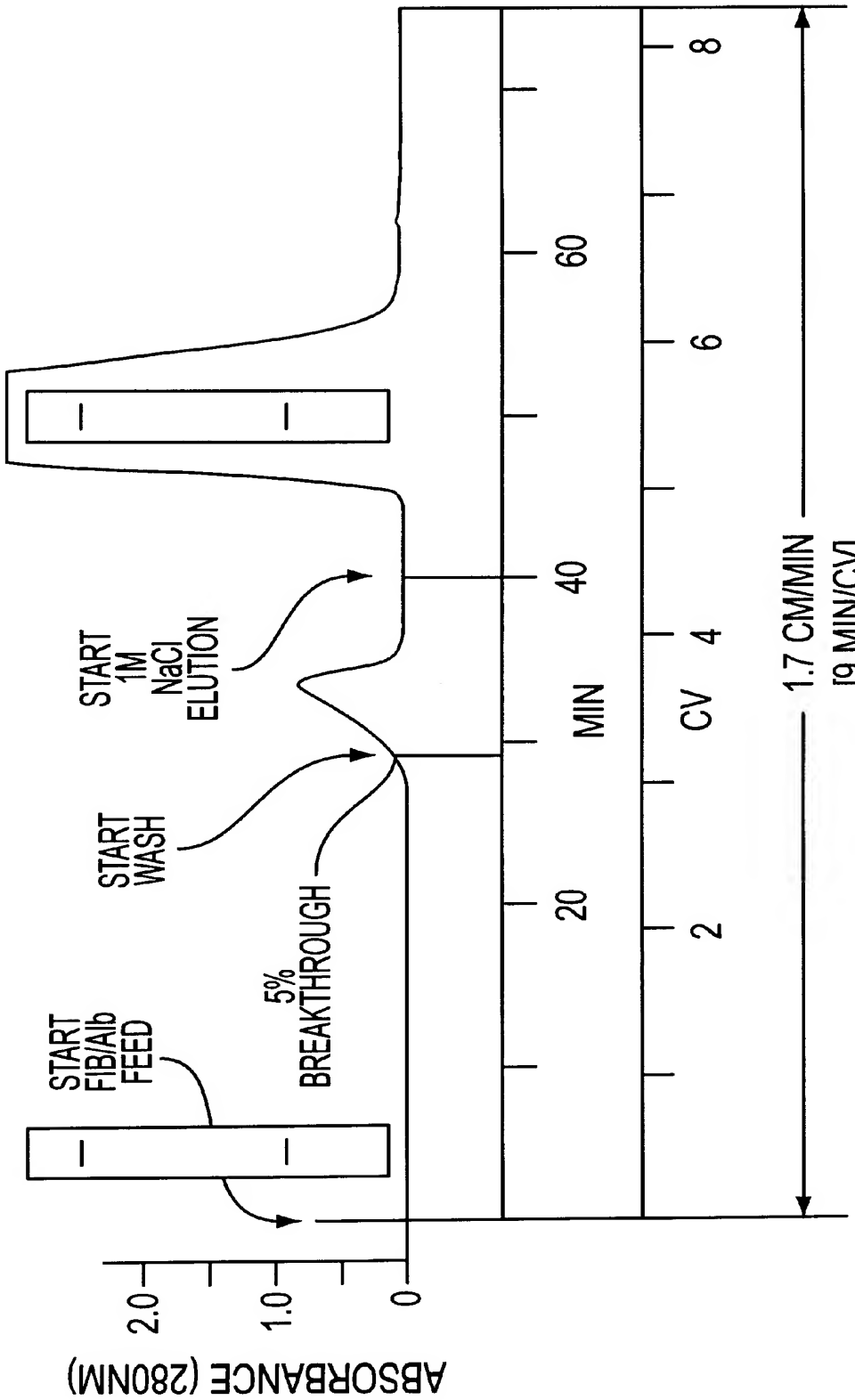

ABSORBANCE (280NM)
2.0
1.0
0
START FIB/Alb FEED
5% BREAKTHROUGH
START WASH
START 1M NaCl ELUTION
20
40
60
MIN
2
4
6
8
CV
1.7 CM/MIN
[9 MIN/CV]
ΔP = 2.7 PSI

FIG. 29(A)

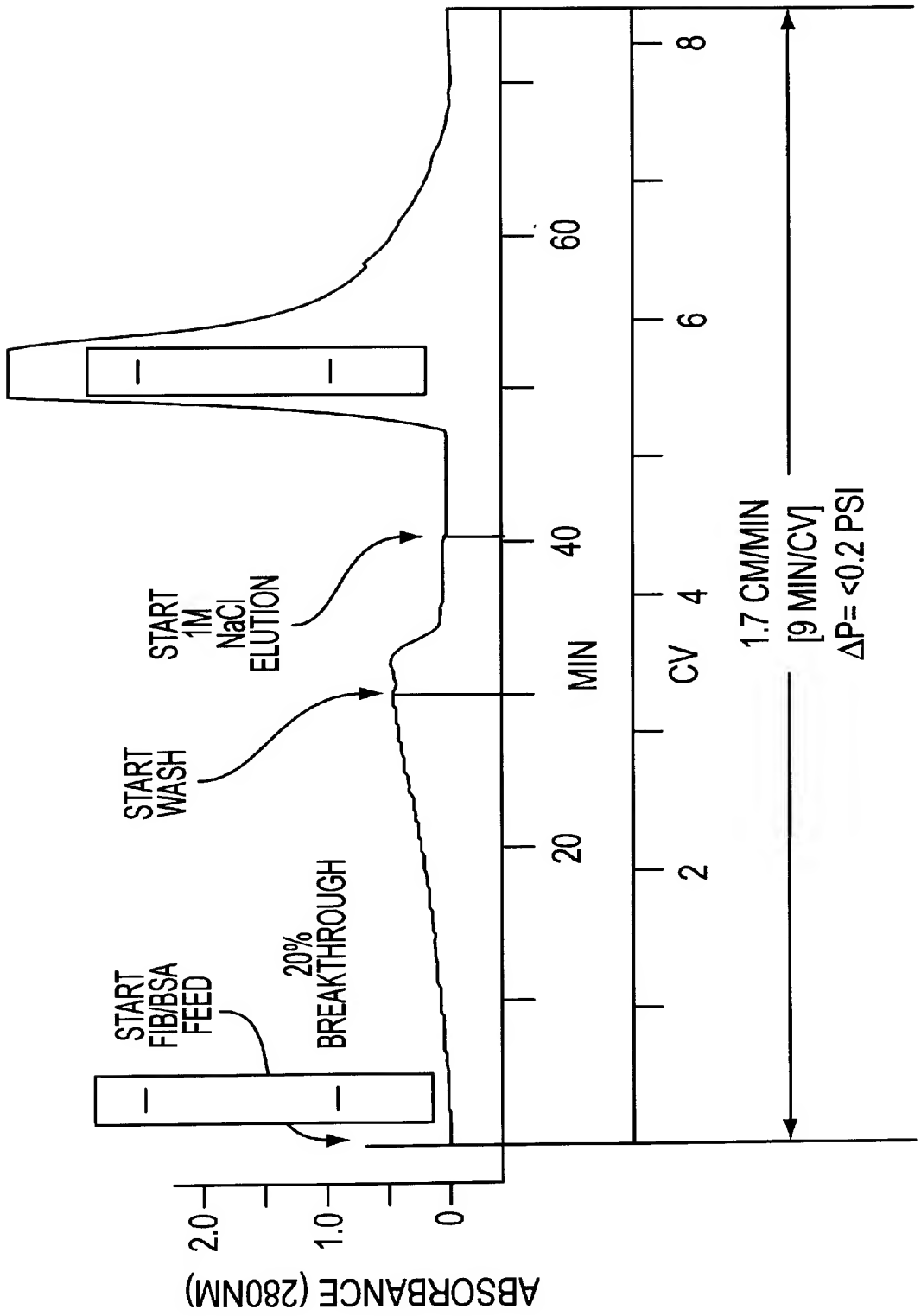

ABSORBANCE (280NM)
2.0
1.0
0
START FIB/BSA FEED
20% BREAKTHROUGH
START WASH
START 1M NaCl ELUTION
20
40
60
MIN
2
4
6
8
CV
1.7 CM/MIN
[9 MIN/CV]
ΔP= <0.2 PSI

FIG. 29(B)

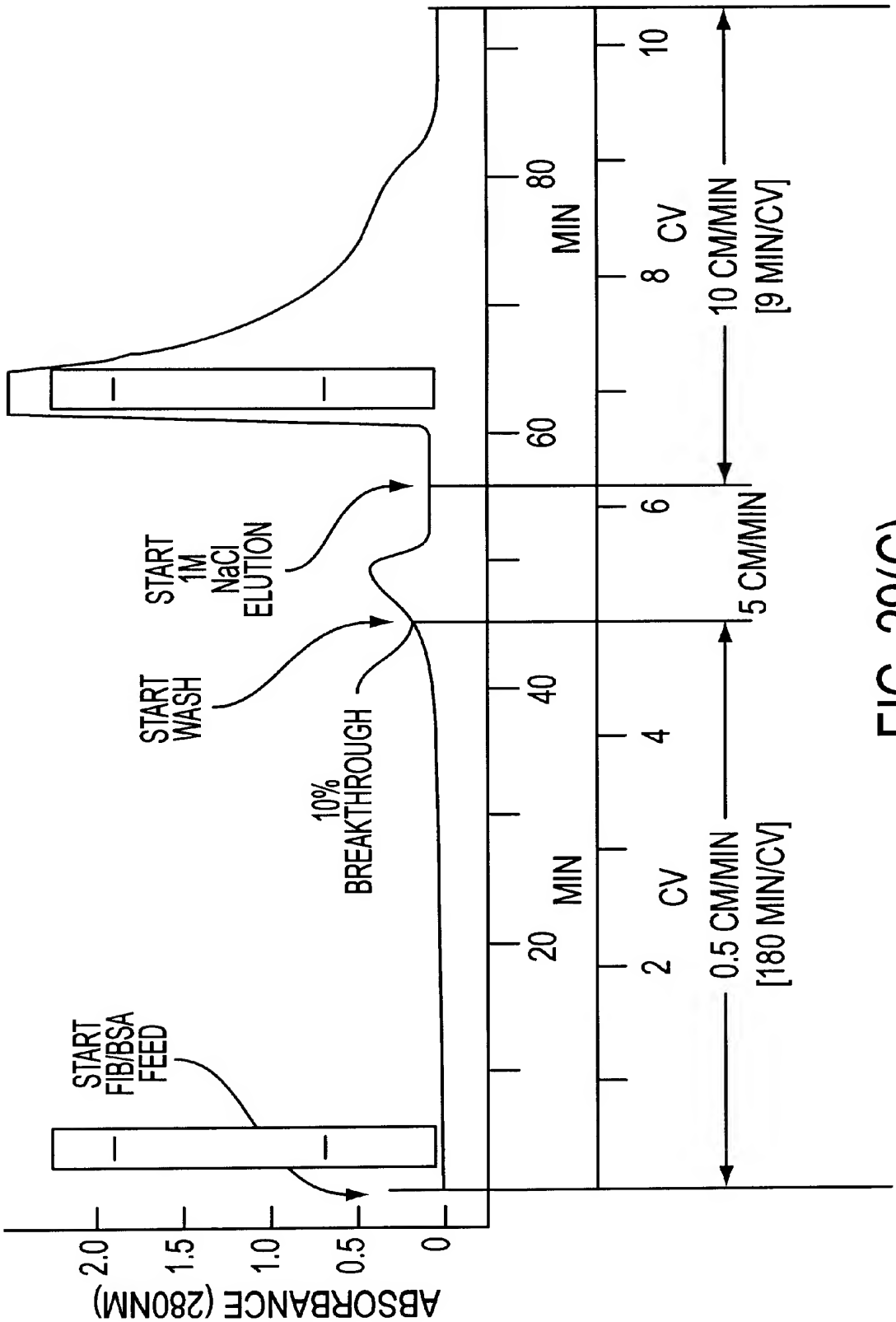

ABSORBANCE (280NM)
2.0
1.5
1.0
0.5
0
START FIB/BSA FEED
10% BREAKTHROUGH
START WASH
START 1M NaCl ELUTION
20 MIN
40
2 CV
0.5 CM/MIN [180 MIN/CV]
4
60 MIN
80
6
5 CM/MIN
8 CV
10 CM/MIN [9 MIN/CV]
10

FIG. 29(C)

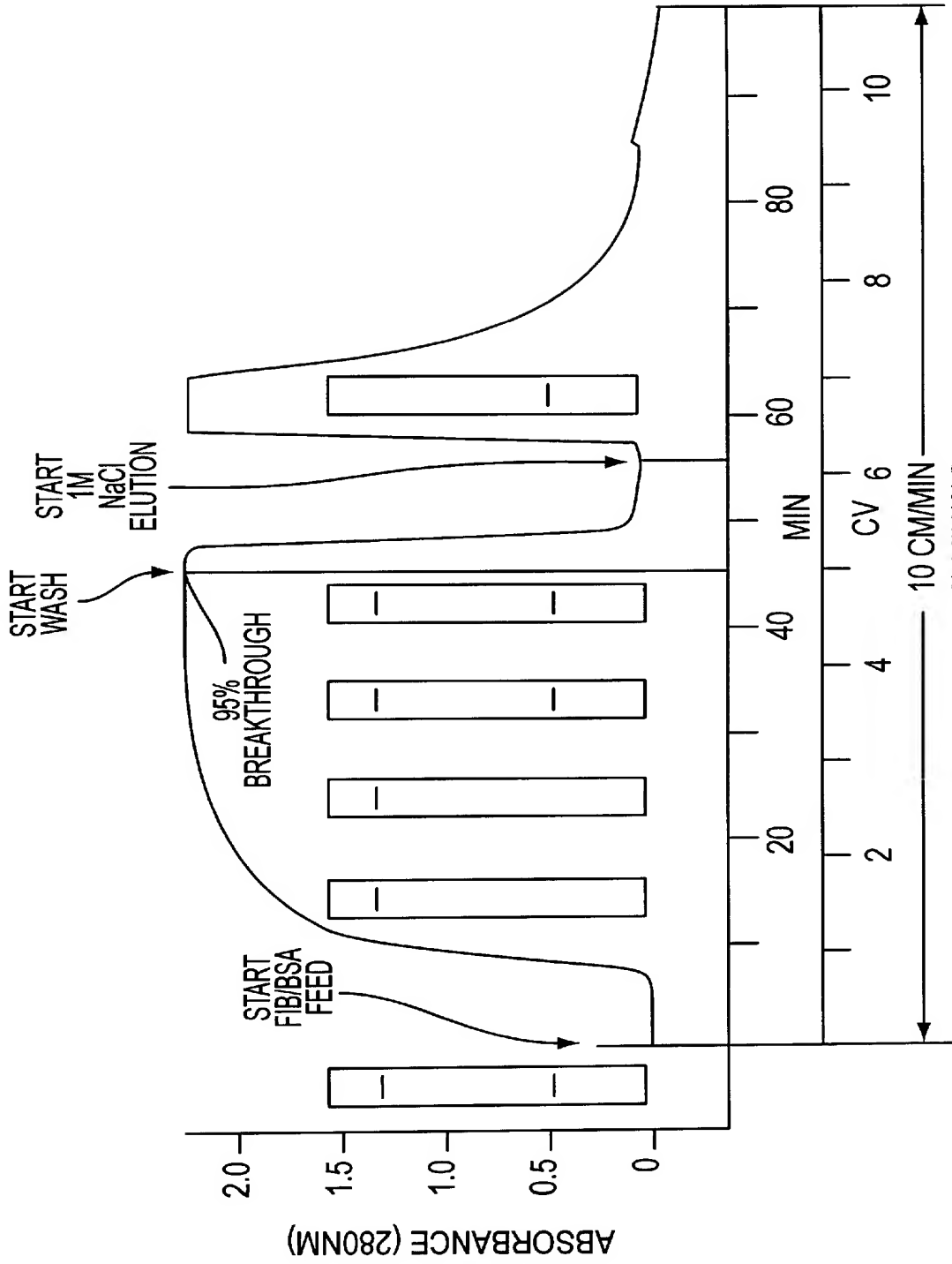

ABSORBANCE (280NM)
2.0
1.5
1.0
0.5
0
START FIB/BSA FEED
95% BREAKTHROUGH
START WASH
START 1M NaCl ELUTION
20
40
60
80
MIN
2
4
6
8
10
CV
10 CM/MIN
[9 MIN/CV]
ΔP=2.7 PSI

FIG. 29(D)

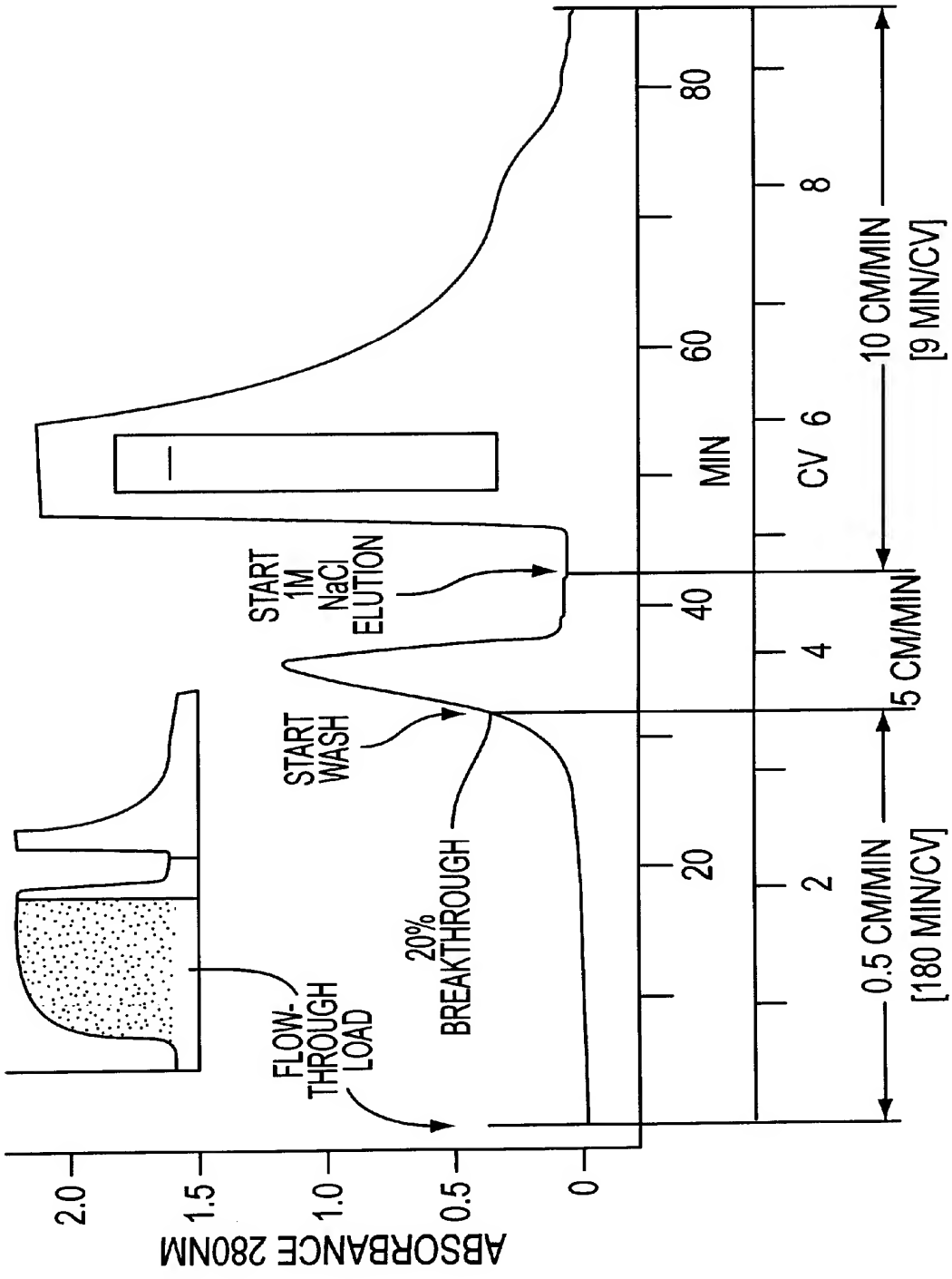

ABSORBANCE 280NM
2.0
1.5
1.0
0.5
0
FLOW-THROUGH LOAD
20% BREAKTHROUGH
START WASH
START 1M NaCl ELUTION
0.5 CM/MIN [180 MIN/CV]
5 CM/MIN
10 CM/MIN [9 MIN/CV]
2
4
CV 6
8
20
40
MIN
60
80

FIG. 29(E)

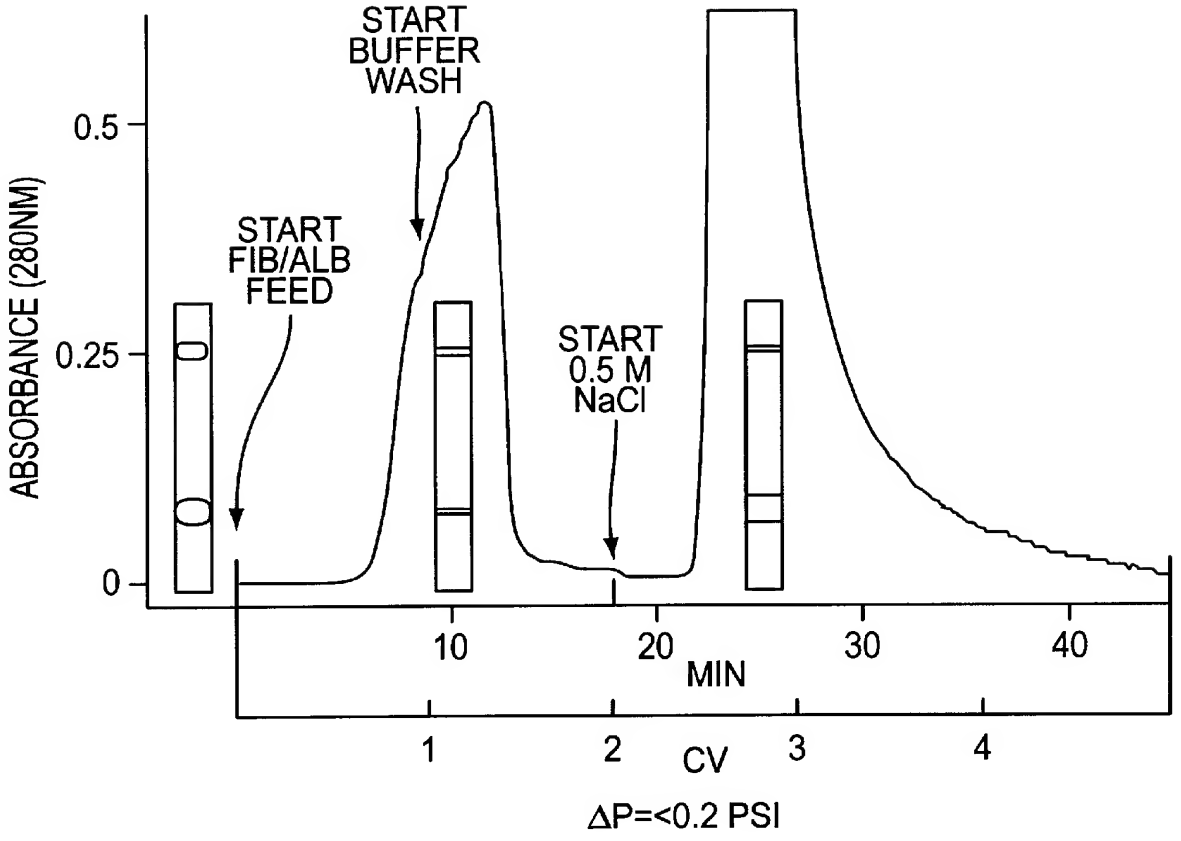

START
BUFFER
WASH
START
FIB/ALB
FEED
START
0.5 M
NaCl
ABSORBANCE (280NM)
0.5
0.25
0
10
20
30
40
MIN
1
2
CV
3
4
ΔP=<0.2 PSI

FIG. 29(F)

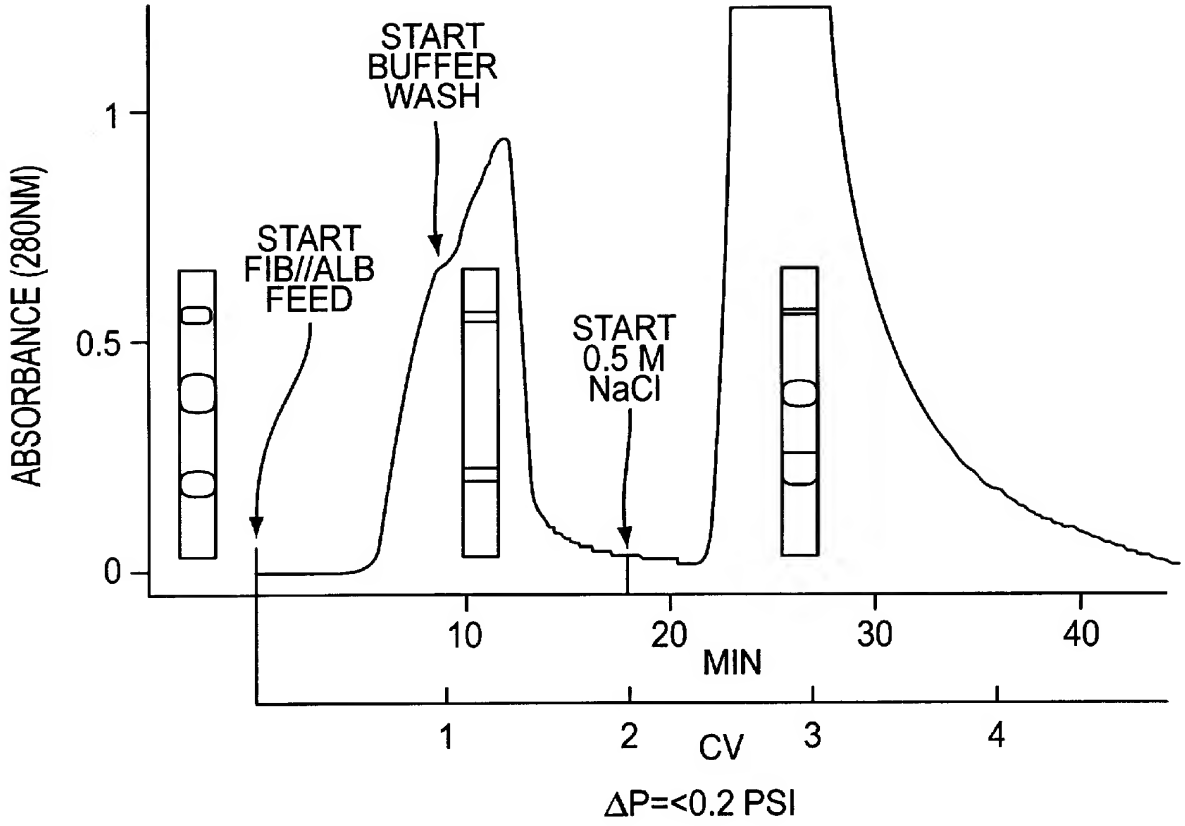

START
BUFFER
WASH
START
FIB//ALB
FEED
START
0.5 M
NaCl
ABSORBANCE (280NM)
1
0.5
0
10
20
30
40
MIN
1
2
CV
3
4
ΔP=<0.2 PSI

FIG. 29(G)

INSIDE-OUT CROSSLINKED AND COMMERCIAL-SCALE HYDROGELS, AND SUB-MACROMOLECULAR SELECTIVE PURIFICATION USING THE HYRDOGELS

FIELD OF THE INVENTION

The present invention relates to chromatographic processes and to ion-exchange and affinity matrices, particularly, to crosslinking of hydrogels made from polymers by inside-out crosslinking and activation methods and to inside-out crosslinked and activated polymeric hydrogels.

The present invention also relates to removal by macromolecular filtration of particles such as viruses and pathogens from virus or pathogen-containing products.

BACKGROUND OF THE INVENTION

Ion-exchange and affinity matrices function based on adsorptive purification processes where the matrix selectively binds a target molecule with greater avidity than other molecules present in the same mixture. Such matrices are used to purify and concentrate proteins and other targets from complex, natural, synthetic, and biosynthetic mixtures. These matrices typically consist of polymeric particles (such as cellulose beads) consisting of a packed bed of particles having void spaces through which liquid can flow. A target molecule solution which is to be purified from solution is passed through the packed bed. Binding sites in the particles constituting the packed bed react with materials to be removed from the complex mixture. Upon passing a washing solution through the column, the eluant leaving the column is a purified target solution. The higher the target binding activity, the higher the purification capacity of the packed bed.

Such ion-exchange and affinity matrices may be constructed from a polymeric hydrogel. A polymeric hydrogel consists of an aqueous part (hence the name "hydro-") and a polymer backbone. Generally, a hydrogel has a low-solids content and is very water-like.

Certain features are peculiar to ion-exchange and affinity matrices, respectively, as set forth below.

When a polymeric hydrogel is to be used as an ion-exchange matrix, the matrix is derivatized to make the matrix ionic. The ionic character of the matrix poses a particular problem, as the matrix tends to dissolve over time and become unusable. Generally, a conventional solution to the dissolution problem has been to crosslink the matrix using conventional crosslinking methods which utilize batch chemistry.

Such conventional methods and matrices suffer from certain disadvantages, discussed further below.

The size (diameter) of the cellulose particles used in constructing the hydrogel influences the properties and performance of the hydrogel. Cellulose particles on the order of 450–600 μm are considered large.

Hydrogels for use on a bench scale (i.e., a small scale) have been available. However, there has been an unsatisfied demand for hydrogels that function on to a larger, commercial scale. For use on such a scale, high through-put is important, that is, the highest possible flow rate for each of the steps in column chromatographic processing when operated at the tallest possible column height (i.e., about 1 m versus 0.1 m). These steps comprise loading (adsorption of target molecules to both surface and intraparticle volume), washing of non-target molecules from the media (matrix), elution of target molecules from the matrix, and cleaning (or regeneration) of the matrix.

Designing large scale adsorption media generally calls into play four considerations, namely, (1) void space pressure driven flow; (2) intraparticle transport; (3) site installation; and (4) media stability.

Void space pressure driven flow refers to the pressure needed to sustain a given flow rate in a packed bed chromatographic column.

Intraparticle transport refers to diffusional and/or convective transport of molecules within the hydrogel particle.

Site installation refers to placing certain pre-ordained structures, so that the sites are not too densely packed and are installed at the desired location within the matrix.

Media stability refers to whether the hydrogel dissolves and/or becomes disintiguous over time, so that it has an acceptable shelf-life or easily deforms under ordinary flow rates used in chromatographic processing.

Traditionally, modifying or designing ion-exchange matrices to be useable on a larger scale than bench-scale, such as for commercial production, has posed difficulties that come from the four aspects mentioned above that often are competing. That is, achieving improved performance on one aspect typically disadvantageously has compromised at least one other aspect. Thus, there is a need for a hydrogel for large-scale use which has satisfactory performance optimized in all four aspects.

The stability problem associated with hydrogels has been addressed by chemical crosslinking, to impart chemical and mechanical robustness and to prevent leaching of polymer backbone into the purified product. However, conventional crosslinking procedures improve stability but at the expense of other aspects of the hydrogel.

Particularly, conventional crosslinking methods are known, whereby, using a crosslinking reagent that generally is a bifunctional molecule, a hydrogel that is "outside-in" crosslinked is produced. In conventional chromatographic hydrogels (e.g. Pharmacia Sepharose Fast-Flow ("FF") crosslinked by the conventional "outside-in" crosslinking procedure, extensive crosslinking occurs near the bead surface before crosslinking occurs in the interior of the bead due to installation by batch chemistry.

Conventional crosslinking molecules (e.g., epichlorhydrin) are insoluble in water, which is the solvent used in the conventional "outside-in" procedure. The conventional water-solvent crosslinking process relies on partitioning the crosslinker into the aqueous phase of the hydrogel, and subsequent reaction with the hydrogel polymer backbone. Such phase partitioning is an inefficient mass transfer operation, and results in little penetration of the crosslinking and/or activating molecule into the interior of the bead prior to the reaction of the cross-linker or activating molecule with the matrix.

As a result, "outside-in" crosslinked hydrogels have a higher degree of crosslinking in the outer strata of the particles, and lower crosslinking in the interior of the particles. Excessive crosslinking at the matrix surface can lessen the accessibility to the interior of the bead, i.e., about 70% of the interior volume becomes inaccessible.

Despite the disadvantages of conventional outside-in crosslinking, abandoning crosslinking is not an acceptable solution, because without crosslinking, the matrix becomes not useable because the matrix dissolves or becomes disintiguous over time (because the polymer becomes soluble when stored or operating in aqueous because the matrix is highly ionic) and/or becomes easily deformed when operated in a chromatographic mode. Shelf-life is an important

consideration for ion exchange applications of hydrogels. Hydrogels with shelf-lives on the order of many months or years, rather than weeks as conventional hydrogels provide, are desired.

Conventional designs of chromatographic matrices emphasized small particle sizes, so as to reduce intraparticle diffusional mass transfer resistance.

Small particle diameters correspond to higher pressure drops, with the use of low L/D (i.e., length-to-diameter) columns to achieve throughout, which is a disadvantage. For the small particles of the conventional hydrogels, high crosslinking becomes necessary because the pressure necessarily will be so high that otherwise the chromatographic media would be deformed. Overall, considerably less crosslinking is needed for large particles in order to provide resistance to deformation while operated under high flow rates and/or tall columns. There is a need for methods to make larger particles usable in hydrogels, because, generally, larger crosslinked-cellulose particles may have certain practical advantages relative to small particles, such as (1) very low pressure at very high linear velocities; (2) allowing for a process with partially clarified, partially filtered feeds; (3) high throughput at large-scale capacity; and, (4) robustness to sanitization. All of the above should be able to occur in a tall bed height without significant pressure drop.

The sanitization point noted above becomes important because most matrices typically are re-used.

Between purification cycles, matrices typically are cleaned with an NaOH solution with pH of about 12–13 at 45° C. for about 2 to 3 hours, which are relatively harsh conditions. The cleaning (and other chemical treatments) can affect the stability of a matrix, by the reagents disrupting hydrogen bonding.

Also, the need for making larger particles usable in hydrogels further corresponds to the relative advantages of manufacturing large compared to smaller particles, of allowing for (1) continuous processing; (2) simplified classification at high yields; (3) ease of manufacturing; and (4) simple manufacturing for product diversity (e.g., crosslinked DEAE (diethyl amino-ethane) cellulose particles; crosslinked Q cellulose particles; affinity-ligand cellulose particles). However, in the conventional methods, the resulting large particles are outside-in crosslinked and correspondingly suffer from certain disadvantages such as (1) lack of accessibility of submacromolecular species to the interior volume of particles by diffusional and convective transport mechanisms due to molecular exclusion or sieving effects (of these submacromolecular species, i.e., proteins, peptides, etc.); and (2) lack of appropriate site installation into accessible intraparticle domains. Thus, there is a need to overcome the disadvantages associated with large conventionally-crosslinked cellulose particles, without giving up any of the advantages that such conventional particles may provide.

The high degree of crosslinking in the art for all particles, small and large, has particularly made large particles unsuitable for ion-exchange and affinity applications at large-scale (i.e., 1 m tall or higher operated at 1 cm/min or greater linear velocity).

For example, the high degree of crosslinking in the outer strata of the conventional large-bead hydrogels results in minimal intraparticle penetration of average sized protein molecules (such as albumin, 66 kDa) at typical large scale processing linear velocities of 1 cm per minute. Thus, less adsorptive capacity in proteins is seen in large particles crosslinked with classical outside-in methods as applied to small particles (i.e., there is a lack of adsorptive surface area where large particles are used). With large beads, overall less surface area is provided, therefore the need to use the bead interior is increased.

Accordingly, in view of the competing considerations discussed above and not satisfactorily addressed by conventional crosslinking and conventional outside-in crosslinked hydrogels, a crosslinking procedure is needed that gives the stability advantages of conventional outside-in crosslinking methods without at the same time suffering from the disadvantages associated with conventional outside-in crosslinking.

In attempting to scale-up ion-exchange matrices (i.e., to design the matrices for larger scale use), one approach has been to use dimensionless group analysis. Dimensionless group analysis uses the governing physics to generate normalized processing parameters which are dimensionless but scale the relative importance of different phenomena to the process (i.e. the ratio of spatial diffusion to a site to the spatial adsorption of the target molecule once that molecule reaches the site). See R. D. Whitley, K. E. Van Cott, and N. H. L. Wang, *Ind. End. Chem. Res.* (1993) 32: 149–159. The Whitley paradigm identified the rate limitations in kinetic and mass transfer steps and allowed for rational scale-up of chromatographic processes based on dimensionless ratios of these rates. Whitley et al. (1993) identified the effects of slow sorption kinetics in multi-component systems.

Intraparticle transport and adsorption kinetics are not well characterized or optimized for most commercially available DEAE matrices. Significant intraparticle transport of proteins at processing scale velocities is absent in commercially-available ion exchange matrices. Void-flow convection and/or surface adsorption kinetics has been identified as the rate limiting mass transfer step for many beaded matrices. Thus, the most important dimensionless group for sorptive (i.e., adsorptive) processes is the adsorption number, N_{+i}, defined by the ratio of sorption kinetic rates to the convection rate. That is,

$$N_{+i} = LC_i k_{+i} / u_o$$

$$N_{-i} = \frac{k_{-i} L}{U_o}$$

where L is the column length; C_i is the concentration of species i; k_{+i} is the adsorption rate coefficient of i; and u_o is the average linear velocity of the fluid in the void space.

Considering the processing variables which affect the N+i expression, it can be seen that column length (L) and linear velocity (u_o, which normalizes volumetric processing rates to the column length to bead and column contacting times) are important in scaling up a chromatographic process from the lab bench to production scale.

Benefits of long column lengths have been recognized. Particularly, with long column length, constant pattern behavior (i.e., steady state plug flow) can be approached. Under conditions of constant pattern the highest possible concentration driving force for adsorptive or desorptive processes occurs. In practical terms, that translates into efficient adsorption and higher capacity; sharper concentration fronts; increased eluted product concentration; less elution and wash volumes; and increased productivity when operated in a long column.

Numerical simulations have shown that for $N_{+i} \geqq 10$, the system can be considered nearly at equilibrium, according to Whitley et al. Under these conditions, the sorption kinetics are not rate limiting and there will be little product loss due

to inefficient adsorption or peak spreading. Thus, efficient chromatographic processes should have high N_{+i} numbers (high ratios of L to u_o) for the adsorption step.

It has been shown experimentally that the N_{+i} variable is the key design variable.

Previously, the present inventors have found that a certain phenomenon governs processing goals and that to optimize scale-up, resolution, and product yields, as well as to develop novel matrices for the isolation of "troublesome proteins", chromatographic processes may be tailored to take advantage of "N". Particularly,

$N_{+i} \geqq 10$ for column loading step

$N_{-i} << 10$ for column washing step

$N_{-i} \geqq 10$ for column elusion step

In the above, i represents any given species (molecule) to be adsorbed, with "+" meaning "adsorption", and "–" meaning "desorption".

Putting the adsorption number theory to work has posed difficulties, because hydrogel matrices for anion exchange adsorption chromatography of proteins frequently incorporate small particles (<100 μm mean particle size) that have a short path length for diffusional transport of target proteins. As a result, high pressure drops, low flow rates, and low L/D column dimensions usually accompany this design emphasis. Thus, there is little room to manipulate $N_{\pm i}$ when doing process scale-up (i.e. increasing column length at constant processing velocity so as to increase $N_{\pm i}$). Large diameter particles (~600 μm) engineered to have minimal intraparticle transport limitations provide the flexibility of high L to u_o and thus maximal N_{+i}.

A model for optimizing affinity media was demonstrated using large diameter (500–700 μm) cellulose particles with relatively low solids contents (Kaster et al., 1994). That optimization yielded an adsorptive media which provided: (i) low pressure drops at high flow rates in a high L/D column mode operation; and, (ii) rapid transport to adsorption sites.

A high L/D column, coupled with the wide range of flow rates available due to minimal pressure drop, allow the user to manipulate the N_{+i} number to a greater extent than for commercially available matrices, would allow for the design of more efficient and productive chromatographic processes. See Whitley et al. (1993); see also J. A. Kaster, W. Oliveira, W. G. Glasser and W. H. Velander, Optimization of pressure-flow limits, strength, intra-particle transport and dynamic capacity by hydrogel solids content and beads size in cellulose immunosorbents, *J. Chromatography* 79–90 (1993).

Thus, a need remains for further methods to optimize hydrogel matrices for large scale protein purification.

Also, conventional matrices would benefit from methods for enhancing ion exchange performance, including methods for exploiting relative rates of mass transfer and sorption kinetics (sorption number $N_{\pm i}$).

Particularly, there is a need for a method of improving the shelf life and deformability of large crosslinked cellulose particles to be used in ion-exchange matrices, especially to bring the shelf-life to the order of many months or years rather than weeks as is the shelf-life for conventionally crosslinked or uncrosslinked ion exchange cellulose particles.

In the case of affinity applications for cellulose particles, there is a need to improve ligand spacing in the cellulose particles.

In affinity applications, getting to the core of the bead is even more important than in the case of ion-exchange matrices, because of the relative number of sites. Sites must be functional. If sites are installed too close together, they will be dysfunctional.

Thus, to summarize the above, there have been many needs for an improved hydrogel and for improved methods of producing hydrogels, particularly for installation of crosslinking or activation chemistries (used to attach affinity ligands).

Additionally, at the same time, in the context of products containing large macromolecular complexes (i.e. particles larger than about 10 nm hydrodynamic radius) such as viruses and other pathogens (e.g., large viral particles such as HIV, Hepatitis B and C) there has been a need for improved methods for selectively removing such pathogens from feedstreams. Existing methods have suffered from various shortcomings, such as loss of valuable feedstream components having hydrodynamic radii of about 5 nm or less (therapeutic proteins).

SUMMARY OF THE INVENTION

A first object of the present invention is to provide an activated matrix which can accommodate and optimize the spatial installation of affinity ligands while preventing the immobilization of excess ligand in the outer strata of the hydrogel bead.

Another object of the present invention is to provide inside outside crosslinked beads (particles) which can be further activated using classical outside in chemistry methods. This classical method should add surface groups at high density. When these groups are quenched with reagent which results in a nonionic group, the surface becomes inert. The particles thus derivatized can be further derivatized with ionic moieties resulting in ionic binding sites primarily within the interior and not on the exterior surface.

Another object of the present invention is to provide a method whereby reaction occurs to a greater extent in the interior of the hydrogel (relative to the outside) to achieve inside-out crosslinking (IOC) or inside-out activation (IOA).

A further object of the present invention is to provide ion exchange cellulose particles with an extended shelf-life, on the order of months or years, and mechanical rigidity (stability) under conditions of flow.

Another object of the present invention is to provide a method for removing macromolecular species, such as HIV, Hepatitis B and Hepatitis C, from submacromolecular species containing such viruses by allowing such macromolecular particles to pass through unadsorbed.

The above objects are achieved by the present invention as described in detail below.

One embodiment of the present invention is a spatial installation method for a bifunctional reagent that crosslinks and/or activates a polymer matrix, comprising at least the step of (a) inside-outside installing a bifunctional reagent on and within a polymer matrix.

In a further embodiment, the polymer matrix of that method comprises at least one cellulose particle.

In another embodiment of the method, the polymer matrix comprises at least one agarose particle.

In an even further embodiment, the polymer matrix comprises at least one chitosan particle.

A further embodiment provides the polymer matrix which comprises a composite of cellulose, agarose, chitosan, and/or other polymer particles.

In an embodiment of the inventive method, the inside-outside installation step comprises (i) spatially distributing the bifunctional reagent throughout the intraparticle volume of the polymer matrix.

The spatial distributing is by a column loading method in one embodiment.

In another embodiment of the present invention, the spatial distribution of the bifunctional reagent is followed by (ii) reacting the polymer matrix with the bifunctional reagent under conditions and for a time to react one functionality of the bifunctional reagent with the polymer matrix.

A further embodiment of the present invention includes the further step of removing the reagent from the void volume of the polymer matrix prior to the reacting step (ii).

In another embodiment of the invention, the reacting step (ii) is followed by the inside-outside crosslinking step of: (iii) further reacting the matrix so to crosslink the matrix, wherein a higher local concentration of crosslinking occurs in the intra-particle volume relative to the local concentration near the outer surface of the matrix.

In a further embodiment of the inventive method, the reacting step (ii) is followed by the inside-outside ligand attachment step of: (iii*) further reacting the matrix with a ligand or an ionic group so that a higher concentration of ligand or ionic moiety occurs on the intra-particle volume relative to the outer surface of the matrix.

Another embodiment of the invention provides, prior to the crosslinking step (iii) or (iii*), a step of classifying by fluidizing.

In another embodiment, there is provided a method for providing inside-out crosslinking of a polymer bead, comprising the steps of: (a) preloading a column of polymer beads with an organic solvent to give a non-aqueous bead/organic solvent preload; (b) adding a bifunctional reagent dissolved in an organic solvent mixture to the bead/organic solvent preload of step (a), to give a bead/organic solvent/bifunctional reagent mixture; and optionally (c) draining excess mixture from void spaces of the bead/organic solvent/bifunctional reagent mixture of step (b).

Another embodiment of the present invention provides a method of keeping a ligand in a hydrogel polymer matrix interior during crosslinking, comprising adjusting solvent conditions during crosslinking.

Another embodiment of the present invention provides a ligand-solution purification method comprising delivering a ligand-solution to a purification column comprising a plurality of inside-out crosslinked particles having binding capacity distributed with more than 90% of the binding capacity in the particle interior and 10% or less on the exterior surface of the particle.

In an embodiment of the inventive ligand-solution purification method, the ligand is a protein.

The present invention also provides an inside-out crosslinked particle, comprising a particle having binding capacity distributed with more than 90% of the binding capacity in the particle interior and 10% or less on the exterior surface.

Another embodiment of the present invention provides an inside-out crosslinked particle, wherein the particle is selected from the group consisting of cellulose, agarose, chitosan and mixtures of two or more of cellulose, agarose and chitosan.

In a further embodiment of the present invention, the inside-out crosslinked particle has a diameter within the range of about 400–600 μm.

Another embodiment of the present invention provides a hydrogel comprising a plurality of inside-out crosslinked polymer particles.

In a further embodiment of the present invention, there is provided an ion-exchange matrix comprising a hydrogel comprising a plurality of inside-out crosslinked polymer particles.

The present invention, in an even further embodiment, provides an affinity matrix comprising a hydrogel comprising a plurality of inside-out crosslinked polymer particles.

In another embodiment of the present invention, a method is provided for further derivativizing an inside-out crosslinked hydrogel comprising a polymer backbone, comprising the step of attaching a protein binding ligand to the polymer backbone.

In a further embodiment of the above inventive method for further derivatization, the protein binding ligand is selected from the group consisting of DEAE, a synthetic or phage display derived polypeptide, and synthetic organics from combinatorial libraries, quartenary ethyl amino ethane ("QEAE"), carboxy methyl ("CM"), a reactive dye, and an antibody.

The present invention also provides a hydrogel polymer crosslinking method comprising (a) inside-out crosslinking a hydrogel polymer, followed by (b) outside-in crosslinking the inside-out crosslinked hydrogel polymer of step (a).

The present invention also provides a hydrogel crosslinking method wherein the outside-in crosslinking step (b) reduces the number of adsorption sites on the bead outer edge.

The present invention further provides a crosslinking method, wherein after the outside-in crosslinking step (b), large particles (i.e., large molecules or large molecular assemblies) in a biological feed source undergo no or lessened non-specific adsorption on or near the matrix outer surface.

In one embodiment of the method of the present invention, the large particles are pathogens.

In another embodiment of the method of the present invention, the large particles are virus particles.

The present invention, in another embodiment, provides a method for producing a commercial-scale hydrogel, comprising: (a) inside-out crosslinking of polymer particle; and (b) constructing a hydrogel comprising the inside-out crosslinked particles of step (a).

Also, one embodiment of the present invention is a commercial-scale hydrogel produced according to the inventive method.

In a further embodiment, the present inventors have provided a method for producing a large-scale purification column, comprising: (a) inside-out crosslinking of polymer particles; and (b) constructing a column comprising the inside-out crosslinked particles of step (a).

In another embodiment, the present invention provides a column produced according to the inventive method, wherein the column has a column length of 90 cm or greater.

In a further embodiment, the present invention provides a method of viral reduction of a virus-containing product, comprising applying a crosslinked hydrogel polymer according to the invention.

In a preferred embodiment of the viral reduction method comprises, an amount of HIV, pathogen, Hepatitis C and/or Hepatitis B is removed from the product.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**(A) depicts the chemical structure of a matrix with unreacted bifunctional reagent; FIG. **1**(B) depicts the chemical structure of a matrix reacted with one functionality of the bifunctional reagent; FIG. **1**(C) depicts the chemical structure of a crosslinked matrix crosslinked by the completely reacted bifunctional reagent.

FIG. **2** is a flow-chart depicting a two-step method of ligand installation.

FIG. **3**(A) shows permeation of antibody in the case of classical high density immobilization. FIG. **3**(B) graphically depicts classical high density immobilization within the intraparticle volume.

FIG. **4**(A) shows two-step immobilization at high Mab density. FIG. **4**(B) depicts such immobilization graphically.

FIGS. **5**(A) and **5**(B) are exploded views depicting spatial distribution of activation. FIG. **5**(A) depicts high activation density through the support for classical outside-in activation. FIG. **5**(B) depicts spatially distributed activation by inside-out activation, according to the present invention.

FIG. **6**(A) is a graph depicting pressure drop versus linear velocity, for a comparison of crosslinked, DEAE-cellulose beads. FIG. **6**(B) graphically depicts pressure drop versus linear velocity, for a comparison of different DEAE media.

FIG. **7** graphically depicts transport in 2%, 3.5% and 6% cross-linked-DEAE cellulose beads under nonadsorbing conditions.

FIG. **8** graphically depicts results relating to transport in 2% cellulose beads (600 um), for uncrossslinked (affinity beads), crosslinked beads, and crosslinked-DEAE beads.

FIG. **9** includes graphs showing data for transport in 2%, 3.5% and 6% "native", underivatized (uncrosslinked) cellulose beads, for a 90×1.6 cm column of native (uncrosslinked) beads of 450–600 μ diameter.

FIGS. **10**(A) and **10**(B) are equilibrium isotherms comparing binding to various DEAE matrices, for albumin (FIG. **10**(A)) and for fibrinogen (FIG. **10**(B)).

FIG. **11** is an equilibrium isotherm graph showing static binding of albumin on crosslinked-DEAE-cellulose matrices of different solids content.

FIG. **12** graphically represent relative titrations of DEAE groups on the matrices as compared to typical dynamic binding capacities (DBC) of DEAE matrices at operating velocities of 10 cm/min.

FIG. **13** shows titrations of DEAE groups on 2% cross-linked-DEAE cellulose beads.

FIG. **14** graphically compares titrations of DEAE groups on 2% cross-linked-DEAE cellulose beads having single and triple DEAE derivatization treatments.

FIG. **15** graphically depicts titrations of DEAE groups on 6% cross-linked-DEAE cellulose beads.

FIG. **16** graphically depicts titrations of DEAE groups on 10% cross-linked-DEAE cellulose beads.

FIG. **17** graphically depicts breakthrough loading of serum albumin on 3.5% cross-linked-DEAE cellulose and DEAE Fast-flow Sepharose at 10 cm/min.

FIG. **18** graphically depicts breakthrough loading of serum albumin on 3.5% cross-linked-DEAE cellulose and Q Fast-Flow Sepharose at 10 cm/min.

FIG. **19** shows the effect of column length on dynamic binding capacity, namely, by increasing adsorption number to increase DBC.

FIG. **20** shows the dynamic binding capacities of 2% and 3.5% cross-linked-DEAE cellulose for BSA at varying conductivities (i.e., ionic strengths).

FIG. **21** shows the effect of column length on the DBC of 3.5% cross-linked-DEAE cellulose beads.

FIG. **22** shows an adsorption number analysis, of the effect of column length on DBC.

FIG. **23** graphically depicts media stability by a 15 swelling analysis of hydrogels according to the present invention, as to chemical treatment and effect on pressure-flow.

FIG. **24** is an equilibrium adsorption isotherm for BSA on cross-linked-Q Cellulose and Q Fast-Flow Sepharose.

FIG. **25** includes various equilibrium adsorption isotherms on quarternary ammonium ("Q")-derivatized Q-cellulose matrices.

FIG. **26** also includes various equilibrium adsorption isotherms on quarternary ammonium ("Q")-derivatized Q-cellulose matrices.

FIG. **27** graphically depicts dynamic binding of serum albumin on cellulose beads under optimal binding conditions operating at a linear velocity of 10 cm/minute and in comparison to Q-sepharose media.

FIG. **28**(A) and (B) are graphs of dynamic binding of fibrinogen on 2% crosslinked-DEAE cellulose beads. FIG. **28**(*a*) gives data for 0.5 cm/minute load, with 10 cm/minute wash and elution. FIG. **28**(*b*) gives data for cm/minute loading, washing and elution.

FIG. **29** graphically represents absorbance data for fibrinogen and albumin.

DETAILED DESCRIPTION OF THE INVENTION

Polymer Matrices and Particles

The polymer particles (which may be spherical beads or asymmetrical particles) for use in the present invention include polymer particles. Cellulose, agarose, dextran, chitosan and composites of these are polymer matrices or particles which are hydrogels and they are preferred examples. Cellulose is the most preferred example.

Suitable polymers which can be crosslinked include those with polysaccharide linkages, e.g., agarose, dextran, cellulose.

Suitable particle sizes which can be used are 350 microns to 1,000 microns, preferably 500 microns to 1,000 microns. Large particles, that is, on the order of about 400–600 um, are preferred. Large particles provide the advantage of producing less back pressure to flow.

Sizing of polymer particles as is discussed herein is known in the art. Particle size may be determined by placing a sample of particles on a glass depression slide and mounting the slide under the microscope. It is preferable to use a video system with a "sizing scale" on the screen. The effective diameter or hydraulic radius of 20 particles from ten different microscopic fields is measured, from which the average bead diameter and standard deviation are calculated.

Preferably, the particles have a generally spherical shape, but it is not necessary for the particles to be perfectly spherical.

The cellulose particles for use in the present invention include cellulose beads which may be commercially obtained, such as cross-linked-DEAE Cellulose Beads (commercially available from Ligochem) and underivatized polymeric matrices such as those made by Pharmacia (e.g., Sepharose Fast Flow, consisting of spherical crosslinked particles less than about 140 microns, made of agarose), Whatman (cellulose, amorphous crosslinked particles for the most part), Biosepra (small composite polymer material, less than 200 microns in diameter), and Sterogene (a large particle, 1 mm in diameter or greater, made of a polysaccharide).

Polymer Particle Preparation

Cellulose particles also may be prepared according to any known method, such as from a cellulose stock solution made from a cellulose powder.

When preparing cellulose particles from cellulose powder, examples of the cellulose powder include CF11 (Whatman; DP 200) and T679 (Weyerhauser; DP 2000).

In preparing a cellulose stock solution for making cellulose beads, in one method, the cellulose first is activated.

In an example of an activation method, weighed-out cellulose is added to deionized water, followed by covering and letting the cellulose swell and absorb the water (for about 5–10 minutes), followed by vigorously mixing (for about 1–2 hours). For vigorous mixing, a paddle-stirring apparatus (100–200 RPM) may be used. A cellulose suspension is thereby formed. The cellulose suspension is filtered through a 10–20μ nylon filter in a vacuum funnel and flask until a "dry cake" is formed. The cellulose cake is removed to a beaker, and dimethylacetamide ("DMAC") is added (e.g., 6–8 volumes of DMAC), followed by mixing (with a spatula) until a uniform cellulose suspension is formed and then vigorously mixing for about 30–60 minutes (using a paddle-stirring apparatus, 100–200 RPM).

The filtering, cake removal, suspension formation and mixing may be repeated, one or more times, to obtain a cellulose cake.

In another method for activating the cellulose, weighed-out cellulose is added to DMAC, covered, and the cellulose allowed to swell for about 1–2 weeks. After that, the cellulose suspension is filtered through a 10–20μ nylon filter in a vacuum funnel and flask until a "dry cake" is formed.

After the cellulose is activated, the cellulose is dissolved. In dissolving the cellulose, a solution of $LiCl_2$ dissolved in DMAC may be used.

In one method, DMAC is poured into a long-necked flask set in a heating mantle. With a paddle-stirring apparatus, the DMAC is vigorously stirred (200–300 RPM). $LiCl_2$ is added to the stirring DMAC. The DMAC/$LiCl_2$ is heated to 80° C. with vigorous stirring. A Thermowatch temperature controller optionally may be used to maintain 80° C. $LiCl_2$ is carefully rinsed from the inside of flask neck using a glass pipette and bulb.

In another method of dissolving the cellulose, a hot (80° C.) DMAC/$LiCl_2$ solution is added to the cellulose cake (in a clean beaker). The hot cellulose solution is stirred with a spatula to give a uniform mixture and then poured into a round-bottom flask containing hot DMAC/$LiCl_2$ solution. Cellulose may be rinsed from the neck of the flask with hot DMAC/$LiCl_2$ solution. The cellulose is stirred (200–300 RPM) at 80° C. for about 2–4 hours. The stirring cellulose solution is covered with foil (around the flask neck which contains the thermometer and stirring paddle) and the mixture is allowed to cool to room temperature for at least 18 hours.

In another method for dissolving cellulose powder, cellulose that was swollen in DMAC for about 1–2 weeks is filtered to a dry cake. The cellulose cake is dissolved, with vigorous stirring, in DMAC containing 8.5% $LiCl_2$ at 80° C. for two hours. The partially dissolved suspension is allowed to completely dissolve by stirring overnight at room temperature (~21° C.).

A stirrer and receiving vessel may be used for washing freshly made beads in distilled water to remove the beading solution.

After the cellulose solution is made, it is beaded. In one method of beading a cellulose solution, an atomizing system is set up by assembling Bete fog nozzle components, including a gasket between the fluid cap and housing, and connecting the "fog nozzle" to the air source and cellulose flow valve (in line with the pressure tank).

The cellulose stock solution may be diluted to give a working solution. The cellulose solution is added to a stainless-steel pressure tank, and the vessel is lock-sealed. The inlet is made of Teflon (trademark of E.I. duPont deNemours & Co.) and is connected to the nitrogen source. The Teflon outlet of the pressure tank/cellulose reservoir is connected to the flow valve (in line with the fog nozzle).

An ethanol/water (50:50 to 80:20 by volume) beading solution is prepared, preferably in an amount about 3 times the volume of cellulose. The beading solution is allowed to de-gas for 5–10 minutes and then poured into the beading tower. A fog nozzle is clamped into place above the beading solution.

After setting up the beading system, the system may be primed. In one priming method, connections in the system (i.e., nitrogen to cellulose tank; cellulose tank to flow valve; flow valve to fog nozzle; fog nozzle to valve to air) are checked and tightened. The clamped fog nozzle is swung over a graduated cylinder to collect the 'priming' fluid. The cellulose tank is pressurized to about 4–10 PSI, and the flow valve is opened to let a stream of cellulose solution flow through the system to remove air and bubbles. The air valve is opened to the fog nozzle and set at 0.3–1.0 PSI.

In one method for beading the cellulose solution, the flowing nozzle is swung back over the ethanol/water beading solution and beads are collected. The beading solution is stirred slowly using a 1–2 inch stirring bar.

The flow of the air and pressure of the tank may be adjusted to get the desired sizes of particles. Preferably, the air pressure is between 0.3–0.5 PSI and the tank (nitrogen) pressure is 5–10 PSI, depending on the concentration (viscosity) of the cellulose solution.

Beading is continued until the cellulose tank is empty, stirring particles for 1–2 hours after beading.

In beading cellulose solutions, nitrogen may be used to pressurize the stainless steel reservoir to force the DRAC/$LiCl_2$ solution through the nozzle assembly from which an aerosol of the cellulose solution is sprayed into the stirring ethanol/water beading solution in a "beading tower" arrangement. Both beads and particles can be made in this way.

After discontinuing the stirring of the bead suspension in the ethanol, the beading solution is allowed to settle for about 3–5 minutes. The ethanol/water (and debris) are decanted off the settled beads.

Beads are poured into a 120 mesh screen pan and rinsed well with de-ionized water, after which the particles begin to become slightly opaque. The rinsed beads then are stored, preferably in about 10 bead volumes of de-ionized water, covered, and stirred vigorously for 18 hours at room temperature.

Polymer Particle Size Sorting

After the cellulose is beaded, the cellulose beads may be sorted by fluidization.

One fluidization method uses a fluidization column, which is made by filling a wash water reservoir with fresh de-ionized water (20 liters) and connecting the appropriate tubing and recycle ports, e.g., Masterflex peristaltic pump with model 7018-52 pump head and tubing #6485-18. Preferably, the top and bottom of the column are fitted with nylon screens (e.g., SpectraMesh 47 mm, 100μ). Wash water is pumped into the inlet (bottom) of the fluidization column at 500 ml/min to remove air from the lines and screen assembly. The column may be filled one-half full and the flow stopped so that the lines and column seals may be

checked for leaks, and bolts evenly tightened if necessary. Up to 25% column volume of beads (~750 ml in a 7.5×75 cm column) are then poured into the top of the column, beads are washed from the top 10 cm of the top and the top screen is assembled. The stopper/screen/retainer plate assembly is bolted evenly around the top. The outlet (top) recycle tubing is connected to the "collection net column" and aligned with the recycle funnel-port on the top of the wash water reservoir.

After column assembly, the beads may undergo expanded bed sorting.

In one method for expanded bed sorting that may be used with medium solids beads, 5–6%, using low speed (about 200–300 ml/min), the column is filled with wash water, the flow connections are checked, the flow is stopped and the beads are allowed to settle for 10–15 minutes. After settling, the bead bed heights are measured and the bead bed is expanded at 500 ml/min (11–12 cm/min) with the top port closed and the side port open, with the collection "net column" connected.

After equilibration at 500 ml/min for 30 minutes, the fine beads are collected and removed from the column to avoid back pressures.

The bead bed is expanded to about 55–60 cm by increasing the flow to ~800 ml/min (18 cm/min). During equilibration with side port collection for about 1–2 hours, the rate of small bead and debris accumulation is monitored, watching for pressure build up in the collection net column. Beads are examined by microscopy to gauge the size range being removed at the flow rate. These beads may be ref luidized to achieve more size classification.

To prepare to collect the target beads, the top port is opened and the side port closed. The side port tubing is removed from the collection net column and placed into a bead collection vessel. The side port is opened and beads are allowed to flow into the collection vessel.

The flow rate may be increased to 1800–2000 ml/min (40–45 cm/min). The flow is adjusted through the top "recycle" port until approximately equal flow occurs from the top port (back into the reservoir) and side port (into the bead collection vessel).

After collecting at 1800–2000 ml/min, the flow is stopped the remaining beads (i.e., the retentate) allowed to settle. The height of the retentate bed is measured or estimated (as often as required if it is less than 1 cm).

The wash water is decanted from the collected beads and the beads pooled into a container. A stock sodium azide solution may be added to bring the beads to a concentration of 0.02%. Bead storage preferably is at 4° C.

In another method of fluidizing cellulose beads, deionized water is pumped ascendingly through beds of freshly made beads at rates from about 4–6 cm/min (for low solids beads) to about 25–45 cm/min (for high solids beads). During equilibration, water circulates through the top of the column and carries the small beads into the collection "net" column (above the reservoir in sink). After the bed is expanded to the appropriate degree, the target beads are collected from the side port into the collection vessel. Two distinct layers are noted within the column: the 'target' beads in the bottom and the small beads (50–400μ) expanding into the upper portion of the column from where they will be collected.

It will be understood that the above methods for producing cellulose beads are by way of example, and that the cellulose beads for use in the present invention are not limited.

It further will be appreciated that other polymer particles may be obtained or made for use in the present invention.

Inside-Outside Crosslinking and Bead Preparation

Observation that inside-outside crosslinking has occurred may proceed as follows.

For a bead according to the present invention that is approximately spherical and 500 microns in diameter, the observable halo is about 50 microns from the particle's edge. The part of the particle (bead) which is heavily crosslinked is about the interior volume from the particle center to about the point where the radius is about 200 microns. Correspondingly, about 50% of the interior bead volume is heavily crosslinked and outer 50% is not heavily crosslinked.

As noted above, the term "inside-outside" crosslinking refers to crosslinking in the interior of the polymer matrix or beads as opposed to crosslinking at the surface of the polymer matrix or bead.

Examples of the exchanging column, into which are placed the polymer beads for carrying out the inside-out crosslinking procedure which is the subject of the present invention, are an XK30 column w/plungers (<500 ml) and a heavy-walled Pyrex column (7.5 cm diameter, fitted with 100μ nylon filters, neoprene stoppers and retainer plates at each end). Other exchanging columns may be used.

The size of the exchanging column may be chosen, depending on batch size. A 5–10% (by volume) headspace is desirable. It is preferable to use a flanged thick-walled pyrex glass cylinder (7.5 cm diameter) ranging in length from 20 cm (~1 1) to 50 cm (~2.5 1), fitted with a butyl rubber screen "assembly" at both ends (bolted together).

The column for use in the present invention includes any column that can accommodate fast flows (e.g., 50 cm/minute or greater in a one-meter column are possible), is compatible with caustic, ethanol and epichlorohydrin, and allows for easy bead removal. As to easy bead removal, preferred configurations include a column with a removable bottom or a column which can be rotated/inverted on the stand (along the clamp axis).

In preparation for crosslinking according to the present invention, the polymer beads are transferred Es into the exchanging column. When the beads are transferred to the exchanging column, preferably head space is left, e.g., 3–5 cm.

After the beads settle in the column, the bed height is measured (and preferably marked on the column).

After measurement of the bed height, the beads optionally are washed before crosslinking.

optionally, a first pre-crosslinking wash may be performed using de-ionized water. An example of the de-ionized water wash is an ascending flow with 3–5 volumes of de-ionized water at 1 column volume/10 min.

The displacement of water from the beads in a column mode by using a dry organic solvent is done before the bifunctional reagent mixture (i.e., a bifunctional reagent (e.g., epichlorohydrin; bis epoxy reagents (e.g., 1,4-butanediol diglycidyl ether); bifunctional oxazoline reagents; bifunctional succinamide based reagents) dissolved in an organic solvent) is introduced to the beads. The bifunctional reagent mixture is introduced preferably in a column mode to the solvent exchanged beads. The reaction of the bifunctional molecule within the beads is done by changing to conditions such as high or low pH, higher temperature, addition of a catalyst or combinations of the above.

The present crosslinking method uses bead bed volumes (bead bed volume being the (measured) volume of a column that has been packed with the particles beads, including the interior void spaces, i.e., the total volume). A preferable reagent for preparing the bead bed volume is epichlorohydrin in ethanol (e.g., 50% epichlorohydrin in 100% ethanol). Epichlorohydrin is virtually insoluble in water but soluble in ethanol.

Preparing more than one bead bed volume is preferable, e.g., two or more bead bed volumes.

After preparing the bead bed volumes, the beads are equilibrated using the bead bed preparation reagent. In a preferred example, 50% epichlorohydrin/ethanol is supplied using ascending flow at 1 column volume per 10 minutes, by pumping. Examples of pumps for use in the present invention include peristaltic pumps (e.g., Masterflex L/S drive peristaltic pump, 10–600 rpm, Cole Parmer, Page 978 in '95/96 catalog); easy load pump heads (used for washing and exchanging beads with solvents and reagents). Examples of tubing for use in the present invention include Viton, Fluran or other chemical resistant tubing #14 (for pumping base) and 16–18 (for washing and exchanging buffers).

After supplying the bead bed preparation reagent (e.g. epichlorohydrin solution), the system is closed off and the beads are allowed to incubate in the bead bed preparation solution, preferably at room temperature for about 30 minutes.

After incubation, interstitial fluid is removed, preferably by reversing the direction of the flow and pumping or drawing air through the column.

After incubation and interstitial fluid removal, the beads are removed to a reaction vessel (e.g., a 3L round bottom flask with top ports).

In the bead removal, preferably a NaOH+0.5% $NaBH_4$ solution is used. In a preferred example, one bead volume of 1 N NaOH+0.5% $NaBH_4$ (5g/l) is used, with a squirt bottle. Optionally, it is preferable to use a heat exchanging device with a thermostat to maintain 25° C.

After bead removal, a bead/NaOH suspension is made. In making the bead/NaOH solution, it is preferable to use 3N NaOH/0.5% $NaBH_4$. In a preferred example, 0.5 column volumes of 3N NaOH/0.5% $NaBH_4$ is prepared in a graduated cylinder and added to the beads.

The bead/NaOH suspension is then stirred. For example, in the reaction vessel containing the removed beads, a paddle-stirring apparatus and pH monitor/controller may be assembled.

An example of the stirrer used for mixing during the various reaction stages in the present invention is a Caframo dual-range stirrer, along with stands and bases (Fisher Sci.).

The pH controller is any pH meter capable of controlling the pumping of NaOH at 10–20 ml/minute at pH 12–13.5, such as a Horizon unit made by New Brunswick and Cole-Palmer.

The bead/NaOH suspension is stirred, keeping the pH above 12.5 until the pH stabilizes at about pH 12.7 to 12.8. In stirring the bead/NaOH suspension, about 100–200 RPM for about 18–24 hours is preferable.

During stirring, the reagent for making the bead/NaOH solution (e.g., 3N NaOH/0.5% $NaBH_4$) is added at a controlled rate, preferably at 10 ml/minute.

To keep the pH above 12.5, it is preferable to continue to add 3N NaOH until the pH "stabilizes" around 12.7 to 12.8 (i.e., no longer decreases), which usually is overnight.

The pH stabilized beads are transferred to the exchanging column and washed. Preferably, washing is with de-ionized water using descending flow, with 3–5 bead bed volumes of de-ionized water at 1 column volume per 10 minutes to remove salts, followed by ethanol washing by descending flow, with 3–5 bead bed volumes of 100% ethanol at 1 column volume per 10 minutes to remove residual epichlorohydrin, followed by de-ionized water washing by ascending flow, with 3–5 bead bed volumes of de-ionized water at 1 column volume per 10 minutes.

After washing and fluid removal, the beads are removed to the reactor vessel. In the removal, it is preferable to use 1 bead volume of 1 N NaOH/0.5% $NaBH_4$ with a squirt bottle.

At this point, the product can be used as an activated matrix. The beads are considered activated at this point where many bifunctional molecules are attached at only one end within the matrix. High temperature attaches the remaining end thus achieving crosslinking.

The beads are stirred (preferably at about 100–200 RPM), and the temperature is slowly raised to 60° C. The initial pH preferably is the range of about 13.0 to 13.4.

Preferably, a Thermowatch temperature controller is used. Advantageously, a paddle-stirring apparatus is used for stirring. A pH monitor preferably is used for monitoring pH.

When the beads have been equilibrated to about 60° C., the pH is adjusted to about pH 13.0, preferably by adding a NaOH/NaBH solution.

Preferably, the NaOH/NaBH solution is added with gentle stirring of the bead suspension at 60° C. for about 18–24 hours. Preferably, 3N NaOH/$NaBH_4$ (~25% bead bed volume) is added once or twice to maintain pH ~13.0 (at setting for 25° C.). The reaction is preferably maintained at pH 13.

After the step at about pH 13.0, the pH is allowed to stabilize to a range of about 12.6 to 12.8. This stabilization preferably occurs overnight, between a second and a third day.

When the pH is stable in the range of about 12.6 to 12.8, the beads are transferred as set forth below.

After pH has stabilized in a range of about 12.7 to 12.8, the beads are washed with water or adjusted to pH<12.

The crosslinked beads may be removed to a storage container.

During the above-described crosslinking reaction, the partitioning of the solvent/crosslinker phase with the external aqueous phase may be observed. The gradual disappearance of the inner solvent/crosslinker phase can be monitored visually during the reaction stage. Fully crosslinked beads have a "halo" appearance, with the highly crosslinked interior of the hydrogel bead differentiated from the sparse outer strata, where the halo extends to about 10–30% from the outer bead diameter inward. That is, for a bead (particle) of about 500 microns in diameter, about the outer 50 microns of the radial dimension constitutes the halo and is lightly crosslinked.

The sparse outer strata of the inside-out crosslinked (IOC) hydrogel has a greater visco-elastic fluid-like property than does the dense more highly crosslinked interior. By environmental electron microscopy, the lack of purely elastic solid structure in the outer strata of the low-solids hydrogels according to the above inventive method is confirmed.

For storing the crosslinked beads, a sodium azide stock solution preferably may be added, to a final concentration of 0.02% sodium azide.

Crosslinked or activated particles prepared according to the above method may be used in a hydrogel in an ion-exchange matrix or an affinity application.

Schematic Representation of Crosslinking/Activated Polymer Matrix or Beads

As shown in FIG. **1**(A), in the starting state, the matrix includes polymer backbone **1** and adjacent polymer backbone **2**. In a first step, bifunctional reagent (molecule) **3** having functionalities **4** and **5** is spatially distributed throughout the intraparticle volume, preferably by column loading methods.

As shown in FIG. **1**(B), the matrix in a second step, is reacted with a bifunctional molecule **3** (so that the reacted polymer backbone **1***a* includes the reacted bifunctional reagent **3***a*) under conditions and timeframe which tend to react one of the functionalities of the bifunctional reagent with the polymer of the matrix while leaving the other functionality **5** unreacted. Preferably, the matrix is activated (reacted) so that the highest concentration of reaction occurs at the center relative to the exterior edge of the particle.

As shown in FIG. **1**(C), next, in a third step, the matrix which is primarily activated/reacted with only one functionality of the bifunctional reagent is further reacted to crosslink the matrix so that more (i.e., a higher concentration of) crosslinking occurs between two polymeric backbones (**1***a* and **2***a*) on the intra-particle volume than on the outer surface.

In an alternative third step, the matrix which is activated/reacted with only one of the functionalities of the bifunctional reagent is further reacted with a ligand or ionic group so that a higher concentration of ligand or ionic moiety occurs on the intraparticle volume than at the outer particle surface.

Additionally, inside-out crosslinked beads according to the present invention may be used in a chromatography station. Preferably, the station consists of long columns (85–95 cm, L/D>50) for cellulose beads, and short columns for Sepharose Fast Flow and Whatman DE-52 beads. Also included in the station are a peristaltic pump with linear velocities of 0.5 to 60 cm/min, a UV detector with a 'fast flow' cell and a computer-interfaced data acquisition hardware and software system.

In another aspect of the invention, a polymer support (preferably a cellulose support) is activated by inside-out crosslinking before ligand attachment.

An example of a support to be activated is a 3.5 wt. % cellulose support.

A preferable example of inside-out activation of a support which is to be used in an affinity application is epoxy-activating the support using the above-outlined inside-out crosslinking method, using epichlorohydrin.

As an example of the beads for use in such a support to be inside-out activated are beads with an average bead diameter of 500 to 600 μm.

Inside-out crosslinked beads prepared according to the invention may be used with ligands such as a monoclonal antibody (e.g., a monoclonal antibody which binds protein C) or a synthetic ligand (e.g., a synthetic ligand which consists of a synthetic peptide which is less than 3500 molecular weight and that binds IgG).

The ligand coupling techniques for use with the inside-out crosslinked beads are not particularly limited. Various affinity ligand coupling techniques in the preparation and the subsequent performance of an inside out ligand attachment (IOLA) based immunosorbent were evaluated.

As an example, a monoclonal antibody (12A8 Mab) directed against recombinant human protein C (rhPC) was used. The cellulose support was epoxy-activated using the above Inside-Out Ligand Attachment technique prior to MAb immobilization as set forth in Example 1.

EXAMPLE 1

A 3.5 wt. % cellulose support was epoxy-activated using the IOLA method using epichlorohydrin. The average bead diameter was 500 to 600 μm. The affinity ligand immobilization methods used were as follows: (1) ligand coupling at constant pH 9.5 using 0.1 M sodium carbonate/0.1 M sodium chloride at 4° C. overnight (classical one-step method); (2) ligand coupling at pH 5.0 for 1 hour in the presence of 0.5 M tris, then adjusted to pH 9.5 and the coupling allowed to proceed overnight at 4° C. (two-step method with nucleophilic competitor); (3) ligand coupling at pH 6.0 for 1 hour, then adjusted to pH 9.5 and the coupling reaction allowed to proceed overnight (conventional two-step method); (4) the cyano-transfer technique (classical one-step method of Kohn et al. (1984); see Kaster et al, J. Chrom, supra.) The column bed volume was 1.0 ml for each case. Under batch-loading conditions, rhPC (1.0 mg/ml) in TBS buffer was batch-equilibrated at 4° C. for 24 hours. Under dynamic-loading conditions, each column was loaded to 200–300% of its maximum theoretical rhPC binding capacity. The bound rhPC was eluted with 2.0 M NaSCN, and rhPC was determined by ELISA.

COMPARATIVE EXAMPLES **1**(a), (b), (c)

As comparisons to Example 1, the affinity ligand immobilization chemistries evaluated included novel two-step immobilization methods, classical one-step immobilization methods, and the classical cyano-transfer technique of Kohn et al. (1984). As to the two-step method, see the flow chart shown in FIG. **2**. This two-step method is concerned with the delivery of ligand to a relatively uniform distribution of an excess of activated sites (FIGS. **2**, **3**(*a*) and **3**(*b*), **4**(*a*) and **4**(*b*)) by altering reaction rates during ligand diffusion into the matrix. This contrasts the alternative strategy of installing activated sites in a gradient from low concentration at the (surface) edge to high concentration within the deep interior of the support. (FIGS. **5**(A) and (B)). The two-step installation can be more optimally achieved when done in combination with an inside-outside activated matrix. The inside-outside method enables one-step classical ligand installation to more effectively install ligand in an active, immobilized state, due to moderated local ligand density.

The reference methods are set forth in G. A. Baumbach and D. J. Hammond, Protein Purification using Ligands Deduced from Peptide Libraries, *BioPharm* (1992) 24–35; E. Boschetti, Review: Advanced Sorbents for Preparative Protein Separation Purposes, *J. Chromatgr.* 658 (1994) 207–236; P. L. Coleman, M. M. Walker, D. S. Milbrath, D. M. Stauffer, J. K. Rasmussen, L. R. Krepski, and S. M. Heilman, Immobilization of Protein A at High Density on Azlactone-Functional Polymeric Beads and Their Use in Affinity Chromatography, *J. Chromatogr.* 512 (1990) 345–363; A. Denizili, A. Y. Rad, E. Piskin, Protein A Immobilized Polyhydroxyethyl-methacrylate Beads.

The % rhPC binding activity of these immunosorbents produced on IOA cellulose beads using either classical one-step or two-step methods of delivering ligand to the bead interior was evaluated under dynamic and batch loading conditions. Ordinary one-step delivery of ligands to classical outside-in activated cellulose beads is also given.

The results for Example 1 and Comparative Examples **1**(a), (b) and (c) were as follows.

The combined use of both the inside-outside ligand attachment (IOLA) epoxy-activation with either two-step or one-step affinity ligand delivery methods provides lower local (spatial) ligand density while concomitantly affording

a satisfactory ligand coupling (immobilization) yield. The results of this study are presented in Table 1, below. A 100% coupling yield was obtained with the classical one-step immobilization using the cyano-transfer technique. This immunosorbent had a density of 10.0 mg mAb/mL support, and an activity of only 0.6% under dynamic-loading conditions. A 31% coupling yield was obtained using the classical one-step method giving a support having a density of 4.8 mg mAb/mL support. The activity for this column under batch-loading conditions was 61%. Similarly, the activity for this immunosorbent under dynamic-loading condition was 50% and 45% for two consecutive chromatographic runs under identical operating conditions. A 50% coupling yield was obtained using the two-step method with the presence of tris as a nucleophilic competitor. This immunosorbent had a density of 7.7 mg Mab/ml support, an activity of 35% under batch-loading conditions, and activities of 38% and 42% under identical dynamic-loading conditions. An 80% coupling yield was obtained using the two-step method without tris present as a nucleophilic competitor. This immunosorbent had a density of 12.4 mg Mab/ml support, and an activity of 26% under batch-loading conditions, and activities of 25% and 30% under identical dynamic-loading conditions.

Inside-out activation according to the invention prespaces the epoxy sites onto the cellulose support to which affinity ligands then can be attached covalently at lower local density. Therefore, the IOLA method should produce a more highly active affinity matrix with either classical one-step or two-step immobilization strategies. The classical one-step immobilization method yielded a lower local Mab density (4.8 mg mAb/mL support) compared to the conventional two-step method (12.4 mg mAb/mL support). However, the classical one-step method provided a higher binding activity under both batch and dynamic-loading conditions compared to the conventional two-step coupling method due to lower local spatial density of immobilized mAb. The lower local spatial density of immobilized mAb decreased stearic hindrance effects, thereby increasing the accessibility of immobilized mAb for the target antigen, rhPC. The presence of tris serving as a nucleophilic competitor in the two-step method gave a lower density (7.7 mg mAb/mL support) compared to the conventional two-step method (12.4 mg mAb/mL support) while providing higher activity under both batch and dynamic-loading conditions. The similarity in binding activity of each of these immunosorbents under both batch and dynamic-loading conditions indicate that there are no mass-transfer limitations involved in the adsorption/desorption kinetics. The immunosorbent prepared using the cyano-transfer technique gave a support with a density of 10.0 mg mAb/mL support, however the binding activity was minimal compared to the classical one-step and both of the two-step methods. The difference in coupling yield between the one-step and two-step methods on IOA-activated matrices likely is due to the cellulose cross-linking which competes with the affinity ligand coupling reaction. The differences in activity likely are due to the accessibility of the high mAb density within the central interior region of the bead.

These results demonstrate the superiority of an IOLA-based support in which the activated epoxy ligand is immobilized from the inside-out of the support where the majority of the epoxy sites are distributed uniformly throughout the matrix interior, compared to an outside-in or predominantly surface installation. The installation of epoxy sites via the IOLA strategy allows an increased uniform affinity ligand density, therefore an opportunity for increased immunosorbent activity. A single one-step ligand delivery can be used because the activation chemistry is already spatially distributed.

Inside-out activation according to the present invention (achieving spatially distributed activation) compared to classical outside-in activation (giving high activation throughout the support) is shown in FIGS. **5**(*a*) (prior art) and **5**(*b*) (present invention).

TABLE 1

Analysis of 12A8 mAb immunosorbent binding efficiency under rhPC dynamic and batch loading conditions.

12A8 MAb immobilization method	% coupling yield	12A8 MAb density (mg/ml support)	% activity (batch loading)	% activity (dynamic loading)
classical 1-step*: pH 9.5 using 0.1 M Na_2CO_3/0.1 M NaCl at 4° C. overnight	31%	4.8	61%	50% 45%
2-step*: pH 5.0 with 0.5 M Tris for 1 hour at 4°, then adjusted to pH 9.5, overnight incubation at 4° C.	50%	7.7	35%	38%–42%
2-step* pH 6.0 for 1 hour at 4°, then adjusted to pH 9.5, overnight incubation at 4° C.	81%	12.4	26%	25% 30%
classical 1-step†: cyano-transfer technique of Kohn et al. (1984)	100%	10.0	—	0.6% 0.6%

(Notes: *3.5 wt % cellulose support activated by IOA method using 50% (v/v) epichlorohydrin/ethanol, †cellulose support activated using classical batch method.)

In a further embodiment of the present invention, inside-out crosslinked beads according to the invention may be derivatized.

In one derivatization method, crosslinked cellulose beads are derivatized using 3 M DEAE as follows.

A DEAE solution is added to the water washed beads, to form a bead/DEAE suspension. DEAE is added (preferably one bead volume of 3M DEAE with mixing). The DEAE solution for derivatizing the beads preferably is 3M DEAE filtered through a 5.0μ membrane cartridge filter. Preferably, the 3 M DEAE is added to the beads, pouring slowly with mixing, washing the bead off of the side of the flask with the DEAE solution.

The bead/DEAE suspension is stirred at for about 1 to 30 minutes at room temperature (~20–23° C.). Preferably, the stirring is slow (about 50–100 RPM) for 30 minutes at room temperature (~23° C.).

NaOH is added to the bead/DEAE suspension. Preferably, NaOH addition occurs while stirring is increased to 250–300 RPM. In a preferred example, using a peristaltic pump (Masterflex, tubing #14), 3 bead volumes of 3N NaOH @ ~1 ml/min/100 ml beads (e.g. 10 ml/minute for 1000 ml beads) is slowly added. Preferably, the reactor port is covered with aluminum foil. Preferably, stirring is continued for about 16–18 hours at room temperature.

On a second day of derivatization, after measuring pH, the beads are transferred to the exchanging column and washed thoroughly, preferably with 5 bead volumes of deionized water using descending flow at 1BV/10 min. When the pH of the wash is <pH 10.0, the beads are equilibrated, preferably with 2 volumes of 1.5M ethanolamine (91.5 ml/liter deionized water, pH=11.5) using ascending flow. At the end of equilibration, the flow is stopped and the beads are incubated in the ethanolamine (in the column) for 4 hours at room temperature.

After incubation, the beads are washed thoroughly, preferably with 5–8 bead bed volumes of deionized water at 1 column volume per 10 minutes using descending flow. When the pH of the wash is <pH 9.0, the column is drained to the top of the bead bed, the exchanging column is disassembled and the beads are removed.

The removed beads may be stored, with sodium azide stock solution added to a final concentration of 0.02% sodium azide.

These beads are chemically stable, anion exchange adsorbants. These beads will be usable as ordinary anion exchange applications where pathogen removal is not critical.

The resulting beads produced by the methods of the present invention may be characterized for various properties.

For example, determining % solids of polymer beads is known. The present inventors determined % solids by the following. For each sample of beads to be measured, three microcentrifuge tubes were labelled and tared to four (4) decimal places (e.g., 1.0000 g). A Kimwipe lab tissue (doubled over) was placed onto a stack of ten paper towels. 5–10 ml of beads were pipetted onto the Kimwipe, spread out with a spatula and the interstitial liquid was allowed to absorb into the paper towels for 15–30 seconds. 1–1.5 ml of "blotted" beads were transferred into the microcentrifuge tubes using a spatula. The tubes were weighed (gross wet weight). The tubes were carefully placed into the "speed vac" lyophilizer. The cover was closed and the centrifuge started. Upon reaching the maximum rotor speed, a vacuum was applied and the samples were freeze-dried overnight. Tubes were removed from the "speed vac" and carefully weighed (to four decimal places) (gross dry weight). The % solids =net dry/net wet, where "net dry"=gross dry−tare and "net wet"=gross wet−tare.

In an example of determining % solids, beads were packed into 'tared' microcentrifuge tubes, in triplicate, and freeze-dried using a "Speed Vac" centrifugal lyophilizer. The tubes were then weighed and the percent cellulose (w/w) that comprises the bead was calculated based on the differences in net-wet and net-dry weights.

Column titrations were performed on the derivatized beads according to the present invention as follows. The recording system and pH flow cell were set. The pH meter was calibrated by pumping pH 10 buffer through the flow cell (adjust pH) followed by pH 4 buffer. A ~10 ml column bed (14–15 cm in the 0.6×20 cm column) was poured. Beads were equilibrated with 40–50 ml 1M NaCl/100 mM NaOH (pH~12.2) @ 2 ml/minute. Eluent was collected in a 100 ml graduated cylinder. The amount of 1M NaCl/100 mM NaOH was recorded and the column height was measured. The recording device/timer was started, along with pumping 10 mM HCl through the column @ 2 ml/min. The eluent was collected in a 100 ml graduated cylinder during the run. At the end of the run (pH~1.8–2.0) the amount of 10 mM HCl used was recorded. Column height was measured. The pH was plotted versus ml 10 mM HCl to give a titration curve. The volume of HCl titrated at pH 6.0 represents the "equivalence" capacity for the beads per ml, or μeq/ml. In an example of a titration set-up for cross-linked-DEAE cellulose beads, beads (10–12 ml) were packed into a 20×0.9 cm column and equilibrated with 1 M NaCl/100 mM NaOH, pH~12.5. HCl (10 mM) was pumped through the bead bed at 2 ml/min passing though a pH electrode flow cell. The pH change was monitored.

Beads according to the present invention also were characterized for their chemical stability.

The effect of 1N NaOH on bed height and titration capacity was studied. After titration of the beads, the column bed height was measured. While the beads were still in the column, the beads were equilibrated with 3–5 column volumes of IN NaOH @ 2 ml/min. Column bed height was measured. The beads were incubated in the column for 16–24 hr. After re-titrating, column bed height again was measured.

Also, the effect of 100 mM HCl (hydrochloric acid) on bed height and titration capacity was studied. After titration of the beads, the column bed height was measured. While the beads were still in the column, they were equilibrated with 3–5 column volumes of 100 mM HCl @ 2 ml/min. Column bed height was measured. The beads were incubated in the column for 16–24 hours. After re-titrating, column bed height was measured.

Next, the % column shrinkage in 4 M NaCl was studied. During the conditioning of the 170–190 ml (85–95 cm) column, prior to the dynamic binding assay, column height was measured before washing with 2 column volumes of 4 M NaCl. After washing the column with 2 column volumes of 4 M NaCl, column bed height was measured. The column was washed with 3–5 column volumes of running buffer and the column bed height was measured.

The present inventors further have studied the pressure stability of the inside-out crosslinked hydrogels according to the present invention.

Pressure studies were done in 1.6×100 cm borosilicate columns (Pharmacia) configured with 100 μm screens at the

bottom. A 90–95 cm cellulose bead column was equilibrated with 50 mM Tris-base, pH 8.3, at 10 cm/min of 3–5 column volumes. The pressure gauge was set to the top of the column using a 3-way valve. Pressure was recorded at zero flow.

For a first run, the flow was started at 5 cm/min, the pressure was equilibrated 1 minutes and the value recorded. Runs were repeated at increasing flows of 10, 15, and 20 cm/min. Column heights were recorded at 20 cm/min. Flow was decreased to zero in 5 cm/min increments, recording the pressures. Column height was measured.

For a second run, the flow was started at 5 cm/min, pressure was equilibrated for 1 minute and recorded. Flow was increased to 10, 15, 20, 25, 30, 35, 40 cm/min, or until 20 PSI was reached. Column heights were recorded at each 5 cm/min increment between 20 and 40 cm/min. Flow was decreased to zero in 5 cm/min increments, recording the pressures. The final column height was measured and the % compression (volume) was determined.

FIG. **6**(A) is a comparison of pressure drops across cross-linked-DEAE cellulose beads. All three beads types were packed in 90 cm×1.6 columns and equilibrated with 5 column volumes of 50 mM Tris-base, pH 8.3 at 10 cm/minute. Compression of the bead bed at 20 cm/min was <1% for all beads. At 40 cm/minute, bed compression was <1% for 10% beads, ~1.2% for 6% beads and ~3% for 2% beads.

FIG. **6**(B) is a comparison of pressure drops across DEAE media. cross-linked-DEAE cellulose beads were packed in a 90 cm column. To achieve comparable linear velocities, Sepharose Fast-Flow and DE-52 were packed in 15 cm×1.6 cm columns. Crushing velocities (i.e., dramatic increases in backpressures) for Sepharose Fast-Flow and DE-52 were at ~20 and 10 cm/minute, respectively. Uncrosslinked native cellulose beads began to crush at linear velocities >20 cm/minute.

The present inventors also have studied transport properties of hydrogels according to the present invention. Transport studies were done in 1.6×100 cm borosilicate columns (Pharmacia) configured with 100 μm screens at the bottom. No screen was used on the top plunger; a ~1 cm headspace was maintained. All tubing connections (i.e. the bottom screen holder and the plunger assembly) were drilled to ⅛ inch and fitted with rigid ⅛×1/16 (ID) PTFE tubing.

A slurry of 180–190 ml of beads in ~400 ml de-ionized water was degassed under vacuum for 5–10 minutes with periodic gentle swirling. The slurry was then poured into the column (held at ~15 degree slant) with the bottom valve open. As the beads packed by gravity and the water flowed out, more degassed bead slurry was added until the packed bed was 90–95 cm. The packed column was conditioned with 5–6 column volumes of 50 mM Tris-base, pH 7.0 at a linear velocity of 10 cm/minute.

Three types of cellulose beads (uncrosslinked, crosslinked and crosslinked/derivatized (cross-linked-DEAE)) with three different cellulose concentrations (% solids) were run with four chromatography standards (dextran blue—Mwt>2,000,000; fibrinogen—Mwt 340,000; bovine serum albumin, BSA—Mwt 60,000; tryptophan—Mwt 204) at three different linear velocities (1.25, 5 and 10 cm/minute) in duplicate (>216 runs).

The buffer delivery system consisted of a peristaltic pump (Masternex 7018–52) with #14 tubing connected to a three way valve at the top of the column through which standards were injected using a 1 ml syringe. To inject the standards, the column flow was stopped and 1 ml of standard was injected directly into the line connected to the column bed. The chromatography was monitored by UV spectrophotometry. Standards were monitored on a Knauer UV Detector at 280 nm. Retention volumes and the peak width (at half peak height) were averaged for each duplicate run and expressed as the fraction of the total column volume.

The linear velocity results are set forth in Table 2 below.

TABLE 2

Beads:	% Solids:	Standards:	Linear Velocities:
1. uncrosslinked	2% cellulose	Dextran Blue (2 mg/ml) mg/ml)	1.25 cm/min
2. crosslinked	6% cellulose	Fibrinogen (5 mg/ml)	5 cm/min
3. cross-linked-DEAE	10% cellulose	BSA (5 mg/ml) Tryptophan (2 mg/ml)	10 cm/min

FIG. **7** shows, for a 90×1.6 cm column of crosslinked and derivatized (DEAE) beads (450–600) according to the present invention, results for transport in 2%, 3.5% and 6% cross-linked-DEAE cellulose beads, by plotting column volume versus UV-absorption at 280 nm. Curves are shown for Dextran Blue, fibrinogen, BSA and tryptophan. The buffer used was 50 mM Tris, pH 7.0 and 0.5 M NaCl. The samples were 1 ml Latex beads (0.3 mm; 1:10); Fibrinogen, 5 mg/ml (FIB); Albumin (BSA), 5 mg/ml; Tryptophan (TRP), 2 mg/ml.

FIG. **8** shows results relating to transport in 2% cellulose beads (600 um), for uncrossslinked (affinity beads), crosslinked beads, and crosslinked-DEAE beads.

FIG. **9** shows data for transport in 2%, 3.5% and 6% "native" (uncrosslinked) cellulose beads, for a 90×1.6 cm column of native (uncrosslinked) beads of 450–600μ diameter. The buffer was 50 mM Tris, pH 7.0 and 0.5 M NaCl. Absorbance at 280 nm was measured. Samples were 1 ml Dextran Blue, 2 mg/ml; Fibrinogen, 5 mg/ml; Albumin (BSA), 5 mg/ml; Tryptophan, 2 mg/ml.

From the data, calculated characteristic diffusion times provide conservative estimates for transport by diffusion. The data show that the actual contacting times are an order of magnitude shorter than predicted by the diffusion time. Thus, the transport in the uncrosslinked beads is likely due to convective mechanisms.

Additionally, the present inventors have conducted pulse-flow transport studies for various-height columns according to the present invention. Tables 3–11 below show pulse-flow transport data for cellulose beads, with column height and crosslinking being varied. The column used was 1.6 cm, with a tris base salt of 7.00. Flow rates of 1.25 cm/min, 5 cm/min and 10 cm/min, respectively, were studied. In each case, beads with diameter 600±150 um were used.

Table 3 below shows data for a 92 cm column, for naked (uncrosslinked) 2% beads. Table 4 contains data for a 93 cm column, for crosslinked 2% beads. Table 5 below shows data for a 91.5 cm, for crosslinked and derivatized 2% beads. Table 6 below shows data for an 89 cm column, for naked 6% beads. Table 7 below shows data for an 85 cm column, for crosslinked 6% beads. Table 8 below shows data for an 90.5 cm column, for crosslinked then derivatized 6% beads. Table 9 below shows data for a 92 cm column, for naked 10% beads. Table 10 below shows data for an 89 cm column, for crosslinked 10% beads. Table 11 below shows data for a 93 cm column, for crosslinked and then derivatized 10% beads. In these Tables, PC is the number of column volume for the peak of the emergent pulse; PW is the pulse width.

The standard deviation is neglected when $\sigma<\pm 0.01$.

TABLE 3

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.1	0.28 (±0.01)	0.76 (±0.11)	0.4 (±0.07)	1.02 (±0.02)	0.55 (±0.1)
Bovine Serum Albumin 5 mg/0.5 ml	1.00 (±0.04)	0.56 (±0.02)	0.65	0.67 (±0.02)	0.52 (±0.03)	0.21
Fibrinogen 2 mg/0.5 ml	0.63 (±0.05)	0.75 (±0.07)	0.38 (±0.01)	0.14 (±0.01)	0.38	0.12
Dextran Blue 5 mg/0.5 ml	0.45 (±0.05)	0.11 (±0.01)	0.37	0.08	0.48 (±0.06)	0.08 (±0.03)

TABLE 4

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.17 (±0.01)	0.37 (±0.01)	1.02	0.43	1.00 (±0.01)	0.52 (±0.02)
Bovine Serum Albumin 5 mg/0.5 ml	0.96 (±0.01)	0.54 (±0.01)	0.6 (±0.01)	0.54 (±0.05)	0.54	0.35 (±0.03)
Fibrinogen 2 mg/0.5 ml	0.48	0.2 (±0.01)	0.43	0.1	0.45	0.11 (±0.03)
Dextran Blue 5 mg/0.5 ml	0.45	0.09 (±0.01)	0.4	0.08	0.4	0.4 (±0.06)

Surprisingly, the fast intraparticle transport is highly retained after inside-outside crosslinking when the penetration of BSA and tryptophan are compared from the results of Table 3 and Table 4.

TABLE 5

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.21 (±0.09)	0.35 (±0.05)	0.98	0.37 (±0.02)	1.02 (±0.02)	0.55 (±0.1)
Bovine Serum Albumin 5 mg/0.5 ml	0.96 (±0.16)	0.53 (±0.04)	0.45	0.35 (±0.02)	0.52 (±0.03)	0.21
Fibrinogen 2 mg/0.5 ml	0.43 (±0.01)	0.1	0.38 (±0.01)	0.07 (±0.01)	0.44 (±0.02)	0.1 (±0.01)
Latex 0.5%	0.45	0.1	0.4	0.1	0.44	0.1

TABLE 6

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.1	0.3	1.01	0.35	1.16 (±0.01)	0.48 (±0.02)
Bovine Serum Albumin 5 mg/0.5 ml	0.86	1.05	0.5	0.24 (±0.01)	0.52 (±0.02)	0.18 (±0.01)
Fibrinogen 2 mg/0.5 ml	0.47 (±0.01)	0.1 (±0.01)	0.43 (±0.02)	0.084	0.48 (±0.02)	0.1 (±0.01)
Dextran Blue 5 mg/0.5 ml	0.53	0.06 (±0.01)	0.45	0.08 (±0.01)	0.48 (±0.01)	0.07 (±0.02)

TABLE 7

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.12 (±0.02)	0.43 (±0.03)	1.00	0.53	1.11 (±0.02)	0.69 (±0.01)
Bovine Serum Albumin 5 mg/0.5 ml	0.57	0.48	0.41	0.19 (±0.01)	0.46 (±0.01)	0.18
Fibrinogen 2 mg/0.5 ml	0.44	0.18	0.4 (±0.02)	0.12	0.41	0.15 (±0.01)
Latex 0.5%	0.43	0.17 (±0.01)	0.41	0.12	0.45	0.16

TABLE 8

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.27	0.55	0.93	0.55 (±0.01)	0.88 (±0.01)	0.71 (±0.07)
Bovine Serum Albumin 5 mg/0.5 ml	0.43	0.2	0.41 (±0.02)	0.12 (±0.01)	0.36 (±0.02)	0.11
Fibrinogen 2 mg/0.5 ml	0.39 (±0.01)	0.1	0.36	0.11 (±0.01)	0.38	0.13
Latex 0.5%	0.42	0.1	0.39	0.04	0.39	0.1

TABLE 9

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.11 (±0.13)	0.47 (±0.05)	0.95 (±0.15)	0.6 (±0.05)	0.96 (±0.07)	0.74 (±0.07)
Bovine Serum Albumin 5 mg/0.5 ml	0.46	0.17	0.4	0.22 (±0.02)	0.46 (±0.02)	0.24 (±0.06)
Fibrinogen 2 mg/0.5 ml	0.4	0.15	0.35 (±0.01)	0.19 (±0.01)	0.39 (±0.01)	0.2 (±0.02)
Dextran Blue 5 mg/0.5 ml	0.45 (±0.07)	0.18 (±0.06)	0.41 (±0.03)	0.14 (±0.04)	0.41 (±0.03)	0.22 (±0.12)

TABLE 10

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.16 (±0.05)	0.32 (±0.05)	0.98	0.37 (±0.02)	1.02 (±0.02)	0.55 (±0.1)
Bovine Serum Albumin 5 mg/0.5 ml	0.43	0.12	0.45	0.35 (±0.02)	0.52 (±0.03)	0.21
Fibrinogen 2 mg/0.5 ml	0.41 (±0.01)	0.08	0.38 (±0.01)	0.07 (±0.01)	0.44 (±0.02)	0.1 (±0.01)
Dextran Blue 5 mg/0.5 ml	0.4	0.07	0.36	0.06	0.38 (±0.03)	0.07 (±0.01)

TABLE 11

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
Tryptophan 5 mg/0.5 ml	1.25 (±0.02)	0.43 (±0.03)	1.03	0.61 (±0.01)	1.03 (±0.01)	0.7
Bovine Serum Albumin	0.46	0.06 (±0.01)	0.42 (±0.02)	0.04 (±0.01)	0.44 (±0.02)	0.04

TABLE 11-continued

	1.25 cm/mn		5 cm/mn		10 cm/mn	
Velocities	PC	PW	PC	PW	PC	PW
5 mg/0.5 ml Fibrinogen 2 mg/0.5 ml Latex 0.5%	0.45 0.42 (±0.01)	0.05 0.06	0.4 0.38	0.054 0.06	0.37 (±0.07) 0.42 (±0.02)	0.032 0.05 (±0.06)

property of the hydrogels according to the present invention, namely, static binding capacity (SBC), by studying adsorption isotherms.

On a first day, stock solutions (~75 ml) were prepared of BSA (or other protein) at concentrations ranging from 0.5 to 50 mg/ml in 50 mM Tris-saline, pH 8.3. OD_{280} of each starting protein solution was measured.

Triplicate 5 ml "snap-cap" tubes for each protein concentration were prepared by carefully pipeting 1 ml of buffer (50 mM Tris-saline, pH 8.3) into each tube.

Beads were washed and equilibrated with 39 mM Tris-phosphate, pH 8.6. (using a 25 mm membrane filter assembly attached to a vacuum flask).

Triplicate samples (1 ml) of cellulose beads were aliquoted into the 5 ml tubes containing 1 ml buffer. After the beads settled, bead "bed" was adjusted to the 1 ml mark and interstitial buffer removed with a pasteur pipet. (With small beads such as Fast-flow Sepharose or Whatman DE-52, brief centrifuging of the tubes (2000–3000×g, 5 min) was done to settle the beads.)

2 ml of each BSA solution/dilution was pipetted to each triplicate set of bead samples, capped tightly and mixed (tumbled) for 18–24 hours at room temperature.

On a second day, the tubes were centrifuged at 2000–3000×g for 5 minutes to settle the beads. 1.5 ml of each supernatant was removed and the residual BSA measured using $O.D._{280}$ [1 mg/ml=0.667].

Isotherm plots were generated. (For such plot generation, a QuatroPro calculation template (or some other spreadsheet) may be used.)

FIG. **10**(A) shows equilibrium isotherms for BSA binding to DEAE matrices. Beads were equilibrated with 39 mM Tris-phosphate, pH 8.6, and triplicate 1 ml aliquots were incubated with 0.5 to 50 mg/ml BSA at room temperature (~21° C.) for 24 h. Concentrations of BSA in the supernatants (C*, mg/ml) were determined by absorption at 280 nm (extinction coefficient; 0.667). Q* (mg/ml) represents the amount of BSA bound to the beads. This figure shows that the Whatman and Sepharose DEAE-derivatized media have essentially equivalent binding capacity for BSA. This is surprising since the 2% DEAE-cellulose beads have much smaller surface area/volume than the Sepharose and Whatman media, yet equivalent or less DEAE moieties.

FIG. **10**(B) shows equilibrium isotherms for fibrinogen binding to DEAE matrices. Beads were equilibrated with 39 mM Tris-phosphate, pH 8.6, and triplicate 1 ml aliquots were incubated with 0.5 to 10 mg/ml fibrinogen at room temperature (~21° C.) for 24 h. Concentrations of fibrinogen in the supernatants (C*, mg/ml) were determined by absorption at 280 nm (extinction coefficient; 1.67). Q* (mg/ml) represents the amount of fibrinogen bound to the beads. The similar amounts of fibrinogen bound is surprising since the DEAE-cellulose beads have so much less surface area/volume than the Whatman and Sepharose DEAE-media.

FIG. **11** is an equilibrium isotherm showing static binding of albumin on DEAE matrices. The beads were equilibrated in the respective buffer and triplicate 1 ml aliquots were incubated with 2 ml of bovine serum albumin (BSA, 0.5 to 50 mg/ml) at room temperature (21° C.) for 24 hours. Concentrations of BSA in the supernatants (C*, mg/ml) were determined by absorption at 280 nm (extinction coefficient, 0.667). Q* (mg/ml) represents the amount of BSA bound to the beads.

Dynamic binding capacity (DBC) was determined by the following procedure.

2–4 liters of running buffer (e.g. 50 mM Tris-HCl, pH 8.3) and 1 liter of 1M NaCl and 4M NaCl, and 1–2 liters of protein solution (e.g., BSA @ 1 mg/ml in running buffer) were respectively prepared.

The beads were diluted in running buffer, degassing the beads under vacuum and packing a one (1) meter column with 85–95 cm of beads (~170–190ml). The column was tapped while the beads settled to remove air bubbles. The column was equilibrated with 3–5 column volumes of running buffer at 5 cm/min.

Protein solution was loaded onto the column until the desired breakthrough (10–50%), recording flows and absorbances. Upon achieving the desired breakthrough, the column was washed with 1–2 column volumes of running buffer (until an even baseline returned). The bound protein was eluted using 1M NaCl until an even baseline returned (up to 2 column volumes). Absorbances and volumes were recorded.

Tables 12 and 13 below show dynamic binding data for cross-linked-DEAE Cellulose and DEAE Ff-Sepharose, for 2% and 3.5% respectively. Table 14 compares dynamic binding of 3.5% cross-linked-DEAE and cross-linked-Q cellulose with DEAE and Q Fast Flow Sepharose at 10 cm/min.

TABLE 12a

2% cross-linked-DEAE cellulose (199)	50 mM TB 100 mM Salt	50 mM TB 50 mM Salt	50 mM TB 35 mM Salt	50 mM TB 20 mM Salt	50 Mm TB
Flow Rate	10 cm/min.	10 cm/min	10 cm/min	10 cm/min	10 cm/min
% Break Through	23% 43%	33% 25%	24%	23% 23%	25% 30%
Conductivity (m)	10.9	5.7	4.6	2.8	0.9
Capacity (mg/ml)	3.0 mg/ml 4.5 mg/ml	15 mg/ml 14.5 mg/ml	15.5 mg/ml	9 mg/ml 8.5 mg/ml	1.5 mg/ml 2.0 mg/ml
% loss Feed	12%, 0.1%	3.1% 3.3%	3%	8.5%, 9.1%	2.2%, 8%
% loss Wash	44%, 34.5%	7.62%, 6.7%	1.5%	1.1%, 1.25%	5.5%, 5%
*(Column Volume/wash)	(12 Column Volumes)	(3 Column Volumes)	(2 Column Volumes)	(2 Column Volumes)	(2 Column Volumes)
% yield Elute	23%, 65.5%	80%, 84%	95%	94%, 87.5%	83%, 81%

Dynamic binding capacities of 2% cross-linked-DEAE cellulose beads using tris buffer (pH 8.6) with varying concentrations of NaCl. Backpressure range, 2–3 PSI

TABLE 12b

2% cross-linked-DEAE cellulose (199)	15 mM Na_3PO_4 100 mM Salt	15 mM Na_3PO_4 50 Mm Salt	15 mM Na_3PO_4 20 mM Salt	15 Mm Na_3PO_4
Flow Rate	10 cm/min.	10 cm/min	10 cm/min	10 cm/min
% Break Through	20.5% 28%	23%	23% 23%	17%
Conductivity (mψ)	10.4	6.0	4.5	3
Capacity (mg/ml)	1.7 mg/ml 1.7 mg/ml	9.6 mg/ml	18 mg/ml 17.1 mg/ml	23 mg/ml
% loss Feed	6.4%, 7%	4.33%	4%, 3.5%	2%
% loss Wash	45%, 60%	20%	5%, 7%	1.3%
*(Column Volume/wash)	(12 Column Volumes)	(5 Column Volumes)	(2 Column Volumes)	(2 Column Volumes)
% yield Elute	42%, 41%	78%	88%, 71%	95%

Dynamic binding capacities of 2% cross-linked-DEAE cellulose beads using sodium phosphate buffer (pH 7.8) with varying concentrations of NaCl. Backpressure range = 2.3 PSI.

TABLE 12c

DEAE-FF Sepharose	15 mM Na_3PO_4 100 mM Salt	15 mM Na_3PO_4 50 mM Salt	15 mM Na_3PO_4 20 mM Salt	15 mM Na_3PO_4
Flow Rate	10 cm/min.	10 cm/min	10 cm/min	10 cm/min
% Break Through	23.6%	20%	47%	20%
Conductivity (m)	9.4	6.1	4.2	2.0
Capacity (mg/ml)	5	14.5	27	35.5
% loss Feed	8%	9.4%	11%	6%
% loss Wash	45%	24%	17%	19%
*(Column Volume/wash)	(15 Column Volumes)	(20 Column Volumes)	(15 Column Volumes)	(8 Column Volumes)
% yield Elute	56%	58%	65%	75%

Dynamic binding capacities of DEAE-FF-Sepharose using 15 mM phosphate buffer (pH 7.8) with varying concentrations of NaCl. Backpressure range = 15–20 PSI.

TABLE 12d

2% cross-linked-DEAE cellulose (199)	40 mM Na_3PO_4 20 mM Salt	40 mM Na_3PO_4	25 mM Na_3PO_4	15 mM Na_3PO_4
Flow Rate	10 cm/min.	10 cm/min	10 cm/min	10 cm/min
% Break Through	27%	23.7%	24%	17%
Conductivity (m)	6.9	5.7	3.3	3
Capacity (mg/ml)	5 mg/ml	7.5 mg/ml	16 mg/ml	23 mg/ml
% loss Feed	4%	6%	3%	2%
% loss Wash	18%	26%	8%	1.3%
% yield Elute	89%	71%	80%	95%

Comparison of the dynamic binding capacities as a function of sodium phosphate concentrations. Backpressure range, 2–3 PSI

TABLE 13

3.5% cross-linked-DEAE cellulose (199)	50 mM TB 100 mM Salt	50 mM TB 50 Mm Salt	50 mM TB 35 mM Salt	50 mM TB, 20 mM Salt	50 Mm TB
Flow Rate		10 cm/min	10 cm/min		10 cm/min
% Break Through		20%	20%		44% (fast break!)
Conductivity (mψ)		6.2	4.4		1.1
Capacity (mg/ml)	Not determined	24 mg/ml	16.8 mg/ml	Not determined	2.2 mg/ml
% loss Feed		2.2%	8%		20%
% loss Wash		6%	<1%		5%
*(Column Volume/wash)		2–3 Column Volumes	(1–2 Column Volumes)		(1–2 Column Volumes)
% yield Elute		91%	92%		75%

Table 13a: Dynamic binding capacity of 3.5% cross-linked-DEAE cellulose beads using tris buffer (pH 8.6) with varying concentration of NaCl. Backpressure range = 2–3, PSI.

3.5% cross-linked-DEAE cellulose (199)	15 mM Na_2PO_4 100 mM Salt	15 mM Na_3PO_4 50 Mm Salt	15 mM Na_3PO_4 20 mM Salt	15 Mm Na_3PO
Flow Rate		10 cm/min		10 cm/min

TABLE 13-continued

% Break Through	22%	23%
Conductivity (mψ)	6.2	2.1
Capacity (mg/ml)	11.5 mg/ml	21.3 mg/ml
% loss Feed	6%	5%
% loss Wash	8.5%	<1%
*(Column Volume/wash)	3–4 Column Volumes	(1–2 Column Volumes)
% yield Elute	85%	95%

Table 13b: Dynamic binding capacity of 3.5% cross-linked-DEAE cellulose beads using sodium phosphate buffer (pH 7.8) with varying concentration of NaCl. Backpressure range = 2–3 PSI.

DEAE-FF Sepharose	15 mM Na_2PO_4 100 mM Salt	15 mM Na_3PO_4 50 Mm Salt	15 mM Na_3PO_4 20 mM Salt	15 Mm Na_3PO
Flow Rate	10 cm/min	10 cm/min	10 cm/min	10 cm/min
% Break Through	23.6%	20%	47%	20%
Conductivity (mψ)	9.4	6.1	4.2	2.0
Capacity (mg/ml)	5	14.5	27	35.5
% loss Feed	8%	9.4%	11%	6%
% loss Wash	45%	24%	17%	19%
*(Column Volume/wash)	(15 Column Volumes)	(20 Column Volumes)	(15 Column Volumes)	(10 Column Volumes)
% yield Elute	56%	58%	65%	74%

Table 13c: Dynamic binding capacity of DEAE FF-Sepharose using 15 mM sodium phosphate buffer (pH 7.8) with varying concentration of NaCl. Backpressure range = 20–25 PSI.

TABLE 14

15 mM Sodium phosphate, pH 7.8 [conductivity 2.1]	3.5% cross-linked-DEAE Cellulose (90 × 1.6 cm)	DEAE Fast-Flow Sepharose (15 × 0.5 cm)	3.5% cross-linked-Q Cellulose (90 × 1.6 cm)	Q Fast-Flow Sepharose (15 × 2.5 cm)
Flow Rate	10 cm/min	10 cm/min	10 cm/min	10 cm/min
% Break Through	23%	20%	20%	21%
Backpressure	2–3 PSI	20–25 PSI	2–3 PSI	20–25 PSI
Capacity (mg/ml)	21/3 mg/ml	35.5 mg/ml	30.5 mg/ml	45.5 mg/ml
% loss Feed	5%	6%	1%	2%
% loss Wash	<1%	19%	2%	10%
*(Column Volume/wash)	1–2 Column Volumes Wash	8–10 Column Volumes Wash	1–2 Column Volumes Wash	20–25 Column Volumes Wash
% yield Elute	56%	58%	65%	74%

In Table 14 above, dynamic binding capacities of 3.5% cross-linked-DEAE and 3.5% cross-linked-Q cellulose beads are compared to DEAE Fast-Flow and Q Fast-Flow Sepharose using 15 mM sodium phosphate, pH 7.8 (conductivity, 2.1) and serum albumin (1 mg/ml) at 10 cm/min.

As to dynamic binding capacity, reference is further made to FIG. **12**, showing relative titrations and dynamic binding capacities of DEAE matrices. to Cellulose beads (10 ml) were equilibrated with 1 M NaCl in 100 mM NaOH and titrated 'in-column' with 10 mM HCl at 2 ml/min as previously described. The dynamic binding capacities (DBC) for bovine serum albumin were determined using optimal buffer conditions at, i) 10 cm/min in 90×1.6 column beds for cellulose beads and ii) 2 cm/min column beds for DE-52 and Sepharose Fast Flow.

FIG. **13** shows titrations of 2% cross-linked-DEAE cellulose beads.

FIG. **14** shows titrations of 2% cross-linked-DEAE cellulose beads with single derivatization, then triple derived. A batch of 2% cellulose beads was derived three times on consecutive days (1 column volume of beads+1 column volume of 3N DEAE+3 column volumes of 1 N NaOH (slow addition) at room temperature) and titrated according to 'SOP'.

FIGS. **15** and 16 show titrations of cross-linked-DEAE cellulose beads, 6% and 10% respectively.

FIGS. **17** and 18 show breakthrough loading of serum albumin on 3.5% cross-linked cellulose and Fast-Flow Sepharose at 10 cm/min for, respectively, 3.5% cross-linked-DEAE Cellulose and DEAE Fast Flow Sepharose, and 3.5% cross-linked-Q Cellulose and Q Fast Flow Sepharose.

FIG. **19** shows the effect of column length on dynamic binding capacity, namely, increasing adsorption number to increase DBC. The dynamic binding capacity of 2% cross-linked-DEAE cellulose beads were determined for BSA at 10 cm/min in 1.6 cm diameter columns (2 ml/cm) of varying lengths, L. The running buffer was 15 mM sodium phosphate, pH 7.8 (conductivity=2.1), and the feed was BSA at 1 mg/ml. Breakthrough was about 20% and protein concentrations were determined by $absorbance_{280}$.

FIG. **20** shows the dynamic binding capacities of 2% and 3.5% cross-linked-DEAE cellulose for BSA at varying conductivities (i.e., ionic strengths). The binding capacities of 2% and 3.5% cross-linked-DEAE cellulose beads (90×1.6 cm bed) for BSA (1 mg/ml) were determined at a linear velocity of 10 cm/min. Operating backpressures ranges from 2–3 PSI for all of the bead beds and all beds washed to baseline within 2–3 column volumes following loading (except for the 3.5% beads in Tris/50 mM NaCl). FIG. **20**(A) is for 15 mM sodium phosphate, pH 7.8 (monobasic+dibasic); 2% cross-linked-DEAE (0, 20, 50 and 100 mM NaCl), 3.5% cross-linked-DEAE (0, 50 and 100 mM NaCl). FIG. **20**(B) is for 50 mM Tris base+HCl , pH 8.6; 2%

cross-linked-DEAE (0, 20, 35, 50, 100 mM NaCl), 3.5% cross-linked-DEAE (0, 20, 35 and 50 mM). When loaded and washed using 50 mM NaCl, the 3.5% beads would not wash below the 20% breakthrough level (the BSA just slowly desorbed in a trial for 10–20 column volumes). However, when loaded in 50 mM NaCl and washed in Tris buffer with no NaCl, the 3.5% beads washed to baseline in 2–3 column volumes and an accurate DBC could be obtained. DBCs with 100 mM NaCl could not be determined accurately on 3.5% beads due to minimal binding and extensive trailing.

FIG. **21** shows the effect of column length on the DBC of 3.5% cross-linked-DEAE cellulose beads. Albumin (bovine serum, 1 mg/ml) was loaded onto 9×5 cm (○) and 90×1.6 ([]) column beds (180 ml) in 15 mM sodium phosphate buffer, pH 7.8, at room temperature to 20–25% breakthrough. After washing to baseline (absorbance 280 nm) with 1–2 column volumes of running buffer, the bound protein was eluted with 1 M NaCl. Beds were reconditioned with 2 column volumes of 4 M NaCl, 1 column volume of 0.5 NaOH and 6 column volumes of running buffer at 10 cm/min.

FIG. **22** shows an adsorption number analysis, off the effect of column length on DBC. 3.5 cross-linked-DEAE cellulose beads, 450–600 u were used. Albumin (bovine serum, 1 mg/ml) was loaded onto 9×5 cm (○) and 90×1.6 ([]) beads (180 ml) in 15 mM sodium phosphate buffer, pH 7.8, at room temperature to 20–25% breakthrough at 1, 2, 5 and 10 cm/ml.

The effect of sanitization on the Dynamic Binding Capacity of 3.5% cross-linked-DEAE and cross-linked-Q Cellulose Beads for Serum Albumin was studied. The columns used were 90×1.6 cm (180 ml) 3.5% cross-linked-DEAE and cross-linked-Q cellulose beads. The sanitization consisted of equilibrating the beads with 2 column volumes of 0.5 N NaOH at 45° C. (using a circulating waterjacket), stopping the flow was stopped and maintaining the column for, (i) 4 hours at 45° C., (ii) 24 hours at 20° C. and (iii) 72 hours at 20° C. After each NaOH sanitization, the column was washed with 6–8 column volumes of 15 mM sodium phosphate buffer, pH 7.8 and the dynamic binding of serum albumin was determined.

Dynamic binding was studied as follows. Bovine serum albumin (1 mg/ml in 15 mM sodium phosphate buffer, pH 7.8) was loaded onto the column at 10 cm/min to ~20% breakthrough. The column was then washed to baseline ($absorbance_{280}$) with 2 column volumes of running buffer, and eluted with 3 column volumes of 1 M NaCl at 10 cm/min. Protein concentrations of the feed, wash and elusion pools were determined by $absorbance_{280}$ The pressure range represents the backpressures from loading to elusion in 1M NaCl.

The results are reported in Table 15 below.

TABLE 15

	3.5% cross-linked-DEAE Cellulose Beads			3.5% cross-linked-Q Cellulose Beads		
Column Treatment	DBC	% Yield	ΔP	DBC	% Yield	ΔP
Before Sanitization	22	92%	2.4–2.9	30.5	96%	2.5–2.9
(0.5 N NaOH, 45C, 4 hours)	mg/ml		PSI	mg/ml		PSI
After Sanitization #1	21.5	94%	2.2–2.9	31	96%	2.4–3.1
(0.5 N NaOH, 45C, 4 hr)	mg/ml		PSI	mg/ml		PSI
After Sanitization #2	21	95%	2.3–2.8	30	95%	2.4–2.9
(0.5 N NaOH, 24 hours, 20C)	mg/ml		PSI	mg/ml		PSI
After Sanitization #3	19	97%	2.4–3.0	29.5	97%	2.6–3.1
(0.5 N NaOH, 72 hours, 20C)	mg/ml		PSI	mg/ml		PSI

It may be seen that a low desorption number, N_{des}, provides a fast washout, in the formula

$$N_{des}=k_{des}L/u_o,$$

where k_{des} is a desorption constant, L is the length of the column, and u_o is the velocity. This N_{des} is the ratio of desorption to convection. Intraparticle mass transfer is not limiting. High k_{des} is countered by high velocity (u).

The present inventors also conducted a swelling analysis of hydrogels according to the present invention, as to chemical treatment and effect on pressure-flow. The results are shown in FIG. **23**. For FIG. **23**, a 90×1.6 cm column bed of 3.5% cross-linked-DEAE beads was (i) flow-packed with 3 column volumes of 15 mM sodium phosphate buffer (NAP), pH 7.8, at 10 cm/min and (ii) conditioned with 1 column volume of 0.5 NaOH at 5 cm/min. Backpressures were measured at the end of steps (a), (c) and (d) in the cleaning cycle. The cleaning cycles included: (a) 4 M NaCl (2 column volumes) at 10 cm/min; (b) 15 mM NAP (2 column volumes) at 10 cm/min; (c) 0.5 N NaOH (1 column volume) at 5 cm/min; and (d) 15 mM NAP (2 column volumes) at 10 cm/min.

FIG. **24** is an equilibrium isotherm for BSA on cross-linked-Q Cellulose and Q Fast-Flow Sepharose. 3.5% cross-linked-Q Cellulose Beads were equilibrated in 15 mM sodium phosphate buffer, pH 7.8, and triplicate 1 ml aliquots were incubated with 2 ml bovine serum albumin (BSA, 0.5 to 50 mg/ml) at room temperature (21° C.) for 24 hours. Concentrations of BSA in the supernatants (C*, mg/ml) were determined by absorption at 280 nm (extinction coefficient; 0.667) Q* (mg/ml) represents the amount of BSA bound to the beads.

Dynamic binding studies were done for cross-linked-Q cellulose and Q FF-Sepharose. The results are set forth in Table 16 below.

Also, dynamic binding of 3.5% cross-linked-DEAE and cross-linked-Q cellulose with DEAE and Q Fast Flow Sepharose at 10 cm/minute was compared. The results are shown in Table 17 below.

TABLE 16

40 mM Tris phosphate pH 8.6 (no salt)	2% Q Beads	3.5% Q Beads	6% Q Beads	10% Q Beads	Q-FF Sepharose
Flow Rate	10 cm/min	10 cm/min	10 cm/min	10 cm/min	10 cm/min
% Breakthrough	31%	20%	17%	13%	42%
Conductivity (mψ)	4.2	4.3	4.2	4.3	4.2
Capacity (mg/ml)	10.4 mg/ml	17.6 mg/ml	16.8 mg/ml	16.5 mg/ml	21.4 mg/ml
% loss Feed	7%	3.5%	5%	3.5	5%
% loss Wash	3%	2%	2%	5%	20%
*(column volumes/wash)	1–2 column volumes	1–2 column volumes	1–2 column volumes	1–2 column volumes	>6 column volumes
% yield Elute	88%	92%	91%	88%	60%

Table 16a: Dynamic binding capacities of cross-linked-Q Beads using 40 mM tris-phosphate (pH 8.6). Backpressure range: Cellulose (90 × .16 cm); 2–3 PSI, Q FF Sepharose (15 × 2.5 cm); 20–25 PSI.

50 mM Tris, pH 8.6 (no salt)	2% Q Beads	3.5% Q Beads	6% Q Beads	10% Q Beads	Q-FFSeph
Flow Rate	10 cm/min	10 cm/min	10 cm/min	10 cm/min	10 cm/min
% Break Through	21%	50%	58%	32%	53%
Conductivity (mψ)	1.0	1.0	1.1	1.0	1.0
Capacity (mg/ml)	10.8 mg/ml	3.9 mg/ml	1–2 mg/ml	1–2 mg/ml	20 mg/ml
% loss Feed	6.5%	25%	16%	4%	3.2%
% loss Wash	8.5%	5%	13%	5%	16.5%
*(column volumes/wash)	2–3 column volumes	1–2 column volumes	1–2 column volumes	1–2 column volumes	1–2 (low MB) volumes
% yield Elute	73%	68%	70%	87%	80%

Table 16b: Dynamic binding capacities of cross-linked-Q Beads using 50 mM Tris buffer (pH 7.8). Backpressure range: Cellulose (90 × 1.6 cm); 20–25 PSI.

15 mM Sod. phosphate pH 7.8 (no salt)	2% Q Beads	3.5% Q Beads	6% Q Beads	10% Q Beads	Q-FFSeph
Flow Rate	10 cm/min	10 cm/min	10 cm/min	10 cm/min	10 cm/min
% Break Through		20%			21%
Conductivity (mψ)		2.1			2.1
Capacity (mg/ml)		30.5 mg/ml			45.5
% loss Feed		1%			2%
% loss Wash		2%			10%
*(column volumes/wash)		2–3 column volumes			25 column volumes
5 yield Elute		96%			88%

Table 16c: Dynamic binding capacities of cross-linked-Q Beads using 15 mM sodium phosphate buffer (pH 7.8). Backpressure range: Cellulose (90 × 1.6 cm); 2–3 PSI, Q FFSeph (15 × 2.5 cm); 20–25 PSI.
[*column volumes/wash, column volumes to elute unbound BSA]

50 mM Tris, pH 8.6 + 100 mM NaCl	2% Q Beads	3.5% Q Beads	6% Q Beads	10% Q Beads	Q-FFSeph
Flow Rate	10 cm/min		10 cm/min	10 cm/min	10 cm/min
% Break Through	24%		23%	23%	48%
Conductivity (mψ)	9.7		9.7	9.8	9.7
Capacity (mg/ml)	5.3 mg/ml		6.8 mg/ml	11.6 mg/ml	27.8 mg/ml
% loss Feed	5%		6.5%	8.5%	3.5%
% loss Wash	16%		27%	5.5%	16.5%
*(column volumes/wash)	3–4 column volumes		>6 column volumes	2–3 column volumes	3–4 column volumes
% yield Elute	73%		68%	92%	80%

Table 16d: Dynamic binding capacities of cross-linked-Q Beads using 50 mM Tris buffer (pH 7.8) + 100 mM NaCl. Backpressure range: Cellulose (90 × 1.6 cm); 2–3 PSI, Q FFSeph (15 × 2.5 cm); 20–25 PSI.

TABLE 17

15 mM Sodium phosphate, pH 7.8 [conductivity 2.1]	3.5% cross-linked-DEAE Cellulose (90 × 1.6 cm)	DEAE Fast-Flow Sepharose (15 × 2.5 cm)	3.5% cross-linked-Q Cellulose (90 × 1/6 cm)	Q Fast-Flow Sepharose (1.5 × 2.5 cm)
Flow Rate	10 cm/min	10 cm/min	10 cm/min	10 cm/min
% Break Through	23%	20%	20%	21%
Backpressure	2–3 PSI	20–25 PSI	2–3 PSI	20–25 PSI
Capacity (mg/ml)	21.3 mg/ml	35.5 mg/ml	30.5 mg/ml	45.5 mg/ml
% loss Feed	5%	6%	1%	2%
% loss Wash	<1%	19%	2%	10%

TABLE 17-continued

15 mM Sodium phosphate, pH 7.8 [conductivity 2.1]	3.5% cross-linked-DEAE Cellulose (90 x 1.6 cm)	DEAE Fast-Flow Sepharose (15 x 2.5 cm)	3.5% cross-linked-Q Cellulose (90 x 1/6 cm)	Q Fast-Flow Sepharose (1.5 x 2.5 cm)
*(column volume/wash)	1–2 column volumes Wash	8–10 column volumes Wash	1–2 column volumes Wash	20–25 column volumes Wash
% yield Elute	95% Yield	75%	95% Yield	88% Yield

Dynamic binding capacities of 3.5% cross-linked-DEAE and 3.5% cross-linked-Q cellulose beads compared to DEAE Fast-Flow and Q Fast-Flow Sepharose using 15 mM phosphate, pH 7.8 (conductivity, 2.1) and serum albumin (1 mg/ml) at 10 cm/min.

Isotherms are shown in FIGS. **25** and **26**.

Table 18 below summarizes dynamic Binding capacities for cross-linked-DEAE cellulose beads.

TABLE 18

Cellulose Beads	Albumin [10 cm/min]	Fibrinogen [10 cm/min]	Fibrinogen [0.5 cm/min]
2% cross-linked-DEAE			
Tris-phosphate	15–20 mg/ml	0.4–0.6 mg/ml	2.7–3.1 mg/ml
Tris-base	4–5 mg/ml	N/D*	N/D
Tris-base + NaCl	4–5 mg/ml	N/D	N/D
6% cross-linked-DEAE			
Tris-phosphate	8–10 mg/ml	<0.2 mg/ml	N/D
Tris-base	1–2 mg/ml	N/D	N/D
Tris-base + NaCl	10–15 mg/ml	N/D	N/D
10% cross-linked-DEAE			
Tris-phosphate	4–6 mg/ml	<0.2 mg/ml	N/D
Tris-base	<0.5 mg/ml	N/D	N/D
Tris-base + NaCl	10–12 mg/ml	N/D	N/D

The dynamic binding capacity of cross-linked-DEAE cellulose beads made with three different cellulose concentrations, 2%, 6% and 10%, were run according to the standard protocol for serum albumin (bovine) and fibrinogen as previously described. The running buffers included, i) 39 mM Tris-phosphate, pH 8.6; 50 mM Tris-base, pH 8.3 and 50 mM Tris-base+100 mM NaCl, pH 8.3. Runs were performed at room temperature (~21° C.). The range of binding capacities represents those obtained between 20–50% breakthroughs.

In another test of the present invention, an experimental binary system was established for albumin and fibrinogen, respectively.

The albumin was present at about 30–60 g/l in blood plasma, and was both a contaminant and a product in plasma fractionation. Albumin has fast ion exchange kinetics and high ion exchange capacity.

The fibrinogen was present at about 2 g/l in blood plasma, and was a high value product in plasma fractionation. Fibrinogen has slow ion exchange kinetics and low ion exchange capacity and is easily denatured and proteolyzed.

The present inventors considered the question of what mechanism is controlling, i.e., film mass transfer, intraparticle mass transfer, convective mass transfer, or adsorption kinetics.

Film mass transfer limitations do not explain the higher capacity of fibrinogen at lower u_o and same column residence time.

As to convection and/or adsorption kinetics, the adsorption kinetics of fibrinogen appear to be relatively slow. However, N_{i+} (L/u_o) does not adequately describe the relationship between the adsorption rate and convection rate. N_{i+} is valid only if L or u_o is kept constant.

In summary, the present invention relating to inside-out crosslinking and inside-out crosslinked cellulose beads provides: low pressure drops at high flow rates for a high L/D column mode; high binding capacity with rapid transport to adsorption sites; and differential N_{i+} allowing for purification by speed.

The dynamic binding capacities of three DEAE media for BSA and fibrinogen were compared. The running buffer for all columns was 39 mM Tris-phosphate, pH 8.6 and all runs were performed at room temperature (~21° C.) with breakthroughs of 20–30%. BSA (1 mg/ml) and fibrinogen (1 mg/ml) was loaded at 10 cm/min, or at the highest linear velocities attainable while maintaining backpressures <20 PSI. Note that at a column length of 15 cm or more, an operating linear velocity of 10 cm/min is not feasible with DEAE-Sepharose FF and Whatman DE-52 due to high back pressures. Table 19 below provides a comparison of dynamic binding capacities (DBC) for DEAE Media.

TABLE 19

DEAE Media [bead length/particle size]	Albumin [high velocity or P]	Fibrinogen [high velocity]	Fibrinogen [low velocity]
2% cross-linked-DEAE Cellulose [90 cm - 500–500 μm]	15–20 mg/ml [10 cm/min, ~3 PSI]	0.4–0.6 mg/ml [10 cm/min, ~3 PSI]	2.7–3.1 mg/ml [0.5 cm/min]
DEAE Sepharose Fast-Flow [15 cm - 50–100 μm]	10–12 mg/ml [7 cm/min, ~20 PSI]	2.2–2.7 mg/ml [7 cm/min, ~20 PSI]	2.4–3.0 mg/ml [0.5 cm/min]
Whatman DE-52 [15 cm - <50 μm]	10–15 mg/ml [2 cm/min, ~20 PSI]	N/D*	3.2–3.9 mg/ml [0.5 cm/min]

Dynamic binding of serum albumin on cellulose beads under optimal binding conditions at 10 cm/minute is reported in FIG. **27**. For FIG. **27**, albumin (1 mg/ml) was loaded onto columns of 2%, 6% and 10% cross-linked-DEAE cellulose beads at 10 cm/min to about 20 breakthrough according to standard procedures. The loading/

running buffer for all columns was 39 mM Tris-phosphate, pH 8.6. Protein concentrations in the pools of the fall-through, wash and 1M salt elution were determined by absorbance at 280 nm. In all cases, the recovery of serum albumin (bovine) was greater than 90%.

Dynamic binding of fibrinogen on 2% crosslinked-DEAE cellulose beads was studied in FIG. **28**. FIG. **28**(*a*) gives data for 0.5 cm/minute load, with 10 cm/minute wash and elution. FIG. **28**(*b*) gives data for 10 cm/minute loading, washing and elution.

Fibrinogen/Albumin were studied by the following methods.

2% cross-linked-DEAE cellulose beads were packed into (i) a 20×5.0 cm or (ii) a 100×1.6 cm borosilicate glass column, washed with 2 column volumes of 4M NaCl, I column volume of 0.5M NaOH and equilibrated with 10 column volumes of 39 mM Tris-phosphate, pH 8.6. Sepharose Fast-Flow (Pharmacia) was packed into a 20 cm×5.0 cm column and conditioned as described above. The buffer delivery system in all cases consisted of a peristaltic pump (Masterflex 7018–52) with #14 tubing connected to a three way valve connected to the top of the column and a pressure gauge. Eluents were monitored by absorbance on a Knauer UV Detector at 280 nm.

Binary models solutions consisted of ~1 mg/ml fibrinogen (FIB, made for ARC by Baxter) and (i) 1 mg/ml, (ii) 5 mg/ml or (iii) 10 mg/ml albumin (ALB, bovine serum albumin, Sigma). That is, three bimodal mixtures were examined with FIB/ALB at ratios of 1:1, 1:5 and 1:10. The loading and running buffer was 39 mM Tris-phosphate, pH 8.6.

Experiments A and B used 1:1 FIB/ALB, ~3.5 column volumes, 15×5.0 cm beds. A 15×5 cm column was packed with 2% cross-linked DEAE beads or Sepharose-FF. These columns were loaded with 1000 ml of a bimodal mixture of FIB/ALB (1 mg/ml each) at 1.7 cm/min, a column residence time of 9 min. The columns were washed with 2 column volumes of tris-phosphate buffer and the bound proteins were eluted with 3 column volumes of 1M NaCl.

Experiment C used 1:1 FIB/ALB, ~5 column volumes, 90×1.6 cm beds. A 90×1.6 cm column was packed with 2% cross-linked DEAE beads (batch #193). This column was loaded with 1000 ml of FIB/ALB (1 mg/ml: 1 mg/ml) at 0.5 cm/min. The column was washed with 1 column volume of tris-phosphate buffer and the bound proteins were eluted with 2 column volumes of 1M NaCl and 2 column volumes of 4M NaCl.

Experiments D and E used 1:1 FIB/ALB, ~5 column volumes, 90×1.6 cm beads. A 90×1.6 cm column was packed with 2% cross-linked DEAE beads (batch #193). This column was loaded with 1000 ml of FIB/ALB (1 mg/ml: 1 mg/ml) at 10 cm/min (Experiment D). The unabsorbed 'flow-through' pool from this 10 cm/min run was then reloaded onto the column (after regeneration) at 0.5 cm/min (Experiment E). In both cases, the columns were washed with 1 column volume of tris-phosphate buffer and the bound proteins were eluted with 2 column volumes of IM NaCl and 2 column volumes of 4M NaCl.

Experiments F and G used 1:5 and 1:10 FIB/ALB, respectively, 1 column volume, 90×1.6 cm beads. A 90×1.6 cm column was packed with 2% cross-linked DEAE beads (batch #193). This column was loaded with i) one column volume (180 ml) of FIB/ALB in a ratio of 1:5 (1 mg/ml:5 mg/ml) at 10 cm/min (Experiment F) or ii) one column volume of FIB/ALB in a ratio of 1:10 (1 mg/ml: 10 mg/ml) at 10 cm/min (Experiment G). In both cases, the columns were washed with one column volume of tris-phosphate buffer and the bound proteins were eluted with 2 column volumes of 1M NaCl and 2 column volumes of 4M NaCl.

In all cases above, the i) 'flow-through' ii) wash, iii) 1M NaCl elusion and iv) 4M NaCl elusion were collected separately and pooled. The volumes and absorbances (280 nm) were measured to determine the mass balance. To regenerate the columns, they were washed with 2 column volumes of 4M NaCl, one column volume of tris-phosphate buffer, one column volume of 0.5 N NaOH and re-equilibrated with 8–10 column volumes of loading buffer.

Table 20 below shows adsorption purification by speed of albumin/fibrinogen mixtures at high L/D and high d_p/u_o, for 2% crosslinked DEAE cellulose beads and DEAE sepharose FF.

TABLE 20

						Fibrinogen		BSA	
Experimental Conditions									
Matrix	Comments	BSA:FiB Feed Ratio	U_o [cm/min]	L [cm]	L/U_o [min]	% FT (Purity)	Purification Factor (Basis)	% Bound (Purity)	Purification Factor (Basis)
2% cross-linked-DEAE Cellulose	Base case for Cellulose Low U_o No separation	1:1 (5 column volumes)	0.5	90	180	5% (100%)	1	100% (51%)	1
2% cross-linked-DEAE Cellulose	Load 5 column volumes of Albumin/Fib High U_o, High L/D Good separation	1:1 (5 column volumes)	10	90	9	85% (80%)	1.6	80% (84%)	1.7
2% cross-linked-DEAE Cellulose	Load 5 column volumes of Albumin/Fib Low U_o, Low L/D	1:1 (3.5 column volumes)	1.6	15	9	30% (100%)	2.0	100% (60%)	1.2
2% cross-linked-DEAE Cellulose	1 column volume of 20% Fib/80% Alb High U_o, High L/D Good Separation	5:1 (1 column volumes)	10	90	9	45% (62%)	3.1	95% (93%)	1.2
2% cross-linked-DEAE	1 column volumes of 10% Fib/90%	10:1 (1	10	90	9	55% (37%)	3.7	90% (95%)	1.1

TABLE 20-continued

Experimental Conditions						Fibrinogen		BSA	
Matrix	Comments	BSA:FiB Feed Ratio	U_o [cm/min]	L [cm]	L/U_o [min]	% FT (Purity)	Purification Factor (Basis)	% Bound (Purity)	Purification Factor (Basis)
Cellulose	Alb High U_o, High L/D Good Separation	column volumes)							
DEAE Sepharose FF	Base Case for DEAE sepharose FF Low U_o, Low L/D No Separation	1:1 (5 column volumes)	1.6	15	9	2%	1	100% (51%)	1
DEAE Sepharose FF	Base Case for DEAE Sepharose FF Low U_o, Low L/D No Separation	1:1 (3 column volumes)	2	18	9	0%	1	100% (50%)	1
DEAE sepharose FF	Load Albumin/Fib at highest possible U_o No separation	1:1 (3 column volumes)	7	15	2	0%	1	100% (50%)	1

FIG. **29** reports results relating to fibrinogen and albumin. The loading/running buffer for all runs was 39 mM Tris-phosphate, pH 8.6.

FIG. **29**-A relates to a DEAE Sepharose Fast-flow-Low L/D column, 1:1. A 15×5 cm bed (300 ml) was loaded at 1.7 cm/min with 1000 ml of fibrinogen (1 mg/ml) and albumin (1 mg/ml). The column residence time was 9 min. Most of the fibrinogen and albumin bound and eluted together. The buffer used was 39 mM Tris-phosphate, pH 8.6.

FIG. **29**-B reports the results for 2% cross-linked-DEAE cellulose beads—Low L/D column, 1:1. A 15×5 cm bed (300 ml) was loaded at 1.7 cm/min with 1000 ml (-3.5 column volumes) of fibrinogen (1 mg/ml) and albumin (1 mg/ml). The column residence time was 9 minutes. Most of the fibrinogen and albumin bound and eluted together.

FIG. **29**-C gives the results for 2% cross-linked-DEAE cellulose beads—High L/D column/Slow load. A 90×1.6 cm bed (180 ml) was loaded at 0.5 cm/min with 1000 ml (~5 column volumes) of fibrinogen (1 mg/ml) and albumin (1 mg/ml). The column residence time was 180 minutes. Most of the fibrinogen and albumin bound and eluted together.

FIG. **29**-D gives the results for 2% cross-linked-DEAE cellulose beads—High L/D column/Fast load. A 90×1.6 cm bed (180 ml) was loaded at 10 cm/min with 1000 ml (~5 column volumes) of fibrinogen (1 mg/ml) and albumin (1 mg/ml). The column residence time was 9 min. Greater than 75% of the fibrinogen flowed through the column without binding, whereas most of the albumin bound and eluted with 1M NaCl.

FIG. **29**-E gives the results for 2% cross-linked-DEAE cellulose beads—Reload. A 90×1.6 cm bed (180 ml) was 're-loaded' at 0.5 cm/min with the 'flow-through' (~650 ml) from the 10 cm/inin FIB/ALB run shown in FIG. **29**-D. The column residence time was 180 minutes. Most of the fibrinogen and remaining albumin bound and eluted together.

FIG. **29**-F provides results for 2% cross-linked-DEAE Cellulose Beads—FIB/ALB 1:5. A 90×1.6 cm bed (180 ml) was loaded at 10 cm/min with 180 ml (1 column volume) of fibrinogen (1 mg/ml) and albumin (5 mg/ml). Most of the fibrinogen 'fell through' the column in a 3.1-fold purification, whereas most of the albumin bound and eluted with 0.5M NaCl.

FIG. **29**-G gives data for 2% cross-linked-DEAE Cellulose Beads—FIB/BSA 1:10. A 90×1.6 cm bed (180 ml) was loaded at 10 cm/min with 180 ml (1 column volume) of fibrinogen (1 mg/ml) and albumin (5 mg/ml). Most of the fibrinogen 'fell through' the column in a 3.7-fold purification, whereas most of the albumin bound and eluted with 0.5M NaCl.

Analysis of 1:5 and 1:10 fibrinogen/albumin was conducted by runs by PAGE. Albumin standards and the eluents of the 1:5 and 1:10 fibrinogen/albumin (FIB/ALB) runs on 2% cross-linked-DEAE cellulose beads (batch 193) were analyzed by PAGE (4–12% gradient gel). Lanes: 1. ALB, 0.2 mg/ml; b. ALB, 0.5 mg/ml; c. 1:5 feed; d. 1:5 flow-through/wash; e. 1:5 NaCl eluent; f. 1:10 feed; g. 1:10 flow-through/wash; h. 1:10 NaCl eluent; i. ALB 1, mg/ml; j. ALB, 2 mg/ml. Concentrations of fibrinogen in the mixtures were determined by ELISA. Concentrations of ALB in the mixtures were determined by laser densitometry of the ALB bands versus ALB (standards e.g., lanes a,b,i, and j). Fibrinogen and BSA yields and purities for a DEAE Sepharose Fast Flow (column length, 15 cm), with loading the fibrinogen or BSA at 1.7 cm/minute, with L/U_o=9 minutes, were as set forth in Table 21 below.

TABLE 21

	Fibrinogen	BSA
Feed	100%	100%
FT & Wash	2%	0%
Elution (purity)	98% (49%)	100% (51%)

Fibrinogen and BSA yields and purities for a 2% DEAE-crosslinked-cellulose bead column (column length, 15 cm), with loading the fibrinogen or BSA at 1.6 cm/minute, with L/U_o=9 minutes. For fibrinogen, feed was 100%, FT and wash was 2%, and elution (purity) was 70% (40%). For BSA, feed was 1005, FT and wash was 0% and elution (purity) was 100% (60%).

Fibrinogen and BSA yields and purities for a 2% DEAE-crosslinked-cellulose bead column (column length, 90 cm), with loading the fibrinogen or BSA at 0.5 cm/minute, with L/U_o=180 minutes, were as follows. Feed was 100% for fibrinogen and BSA; FT & Wash was 5% for fibrinogen and 0% for BSA; elution (purity) was 95% (49%) for fibrinogen and 100% (51%) for BSA.

Fibrinogen and BSA yields and purities for a 2% DEAE-crosslinked-cellulose bead column (column length, 90 cm),

with loading the fibrinogen or BSA at 0.5 cm/minute, with L/U_o=9 minutes for loading for Feed #1 and 180 minutes for loading for Feed #2, were as follows. Fibrinogen had feed #1, 100%; FT and wash 85%; elution #1, 15%; feed #2 (from FT and wash #1), 15 85%; FT and wash #2, 8% and elution #2, 77%, for overall yields (purity) of 77% (79%). BSA had feed #1, 100%; FT and wash 21%; elution #1, 79%, for overall yields (purity) of 79% (84%).

In a preferred embodiment of the present invention, an inside-out crosslinked hydrogel is used in a method for purifying a virus-containing biological product, to thereby remove a pathogen, e.g., HIV, Hepatitis B and/or Hepatitis C.

While the invention is described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that changes and modifications can be made therein without departing from the spirit and scope thereof.

We claim:

1. An inside-outside spatial installation method for a bifunctional reagent that crosslinks and/or activates a polymer matrix, comprising at least the step of:

(a) pre-loading a polymer matrix comprising a column of polymer beads with an organic solvent to give a non-aqueous bead/organic solvent pre-load,

wherein the polymer matrix comprises (i) at least one cellulose particle, (ii) at least one agarose particle. (iii) at least one chitosan particle (iv) at least one dextran particle, (v) at least one polymer particle of a polymer having polysaccharide linkages, or (vi) a composite of cellulose, agarose, chitosan and/or other polymer particles;

(b) to the bead/organic solvent pre-load of step (a), adding a bifunctional reagent dissolved in an organic solvent mixture to give a bead/organic solvent/bifunctional reagent mixture,

(c) for the bead/organic solvent/bifunctional reagent mixture provided in step (b), reacting one functionality of the bifunctional reagent with the polymer matrix.

2. The method of claim **1**, wherein the polymer matrix comprises at least one cellulose particle.

3. The method of claim **1**, wherein the polymer matrix comprises at least one agarose particle.

4. The method of claim **1**, wherein the polymer matrix comprises at least one chitosan particle.

5. The method of claim **1**, wherein the polymer matrix comprises a composite of cellulose, agarose, chitosan, and/or other polymer particles.

6. The method of claim **1**, further comprising removing the reagent from the void volume of the polymer matrix prior to the reacting step (c).

7. The method of claim **1**, wherein step (c) is followed by the inside-outside crosslinking step of:

(d) further reacting the matrix so to crosslink the matrix, wherein a higher local concentration of crosslinking occurs on the intra-particle volume relative to the local concentration near the outer surface of the matrix.

8. The method of claim **1**, wherein step (c) is followed by the inside-outside ligand attachment step of:

(d*) further reacting the matrix with a ligand or an ionic group so that a higher concentration of ligand or ionic moiety occurs on the intra-particle volume relative to the outer surface of the matrix.

9. The method of claim **7**, further comprising, prior to crosslinking step (d), a step of classifying by fluidizing.

10. The method of claim **8**, further comprising, prior to crosslinking step (d*), a step of classifying by fluidizing.

11. A inside-outside spatial installation method for a bifunctional reagent that crosslinks and/or activates a polymer matrix, comprising at least the step of:

(a) pre-loading a polymer matrix comprising a column of polymer beads with an organic solvent to give a non-aqueous bead/organic solvent pre-load;

(b) to the bead/organic solvent pre-load of step (a), adding a bifunctional reagent dissolved in an organic solvent mixture to give a bead/organic solvent/bifunctional reagent mixture,

(c) for the bead/organic solvent/bifunctional reagent mixture provided in step (b), reacting one functionality of the bifunctional reagent with the polymer matrix.

12. The method of claim **1**, wherein the polymer particles are 350 microns to 1,000 microns.

13. The method of claim **12**, wherein the polymer particles are 500 microns to 1,000 microns.

14. The method of claim **12**, wherein the polymer particles are 400 to 600 um.

15. The method of claim **1**, further comprising, before step (a), a step of polymer particle sizing.

16. The method of claim **1**, wherein an approximately spherical bead of about 500 microns is used in step (a),

such that a crosslinked bead is provided with an observable halo of about 50 microns from the particle edge, with the heavily crosslinked part of the bead in the interior volume of the particle sphere from the particle center to about the point where the radius is about 200 microns.

17. The method of claim **1**, wherein, before step (a), a pre-crosslinking wash is performed using de-ionized water.

18. The method of claim **1**, wherein the addition of the bifunctional reagent in step (b) is in a column mode.

19. The method of claim **1**, wherein the step (c) reaction of the bifunctional molecule within the beads is done by changing pH, increasing temperature, catalyst addition or a combination thereof.

20. The method of claim **1**, wherein the organic solvent is epichlorohydrin in ethanol.

21. The method of claim **20**, wherein after the epichorohydrin in ethanol solution is supplied to the beads, the column is closed and the beads are allowed to incubate in the solution.

22. The method of claim **21**, wherein the incubation is at room temperature for about 30 minutes.

23. The method of claim **1**, wherein before step (c), the beads are removed from the column and in step (c), the beads are suspended in a NaOH solution.

24. The method of claim **23**, wherein the bead/NaOH solution is stabilized to about pH 12.7 to 12.8 and stirred at about 100–200 rpm for about 18–24 hours.

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